Skeletal muscle protein content of lipolytic inhibitor G(0)/G(1) switch gene-2 protein: the effect of endurance training

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Abstract

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The first and rate-limiting step of lipolysis is the removal of the first fatty acid from a triglyceride molecule; it is catalyzed by adipose triglyceride lipase (ATGL). ATGL is co-activated by comparative gene identification-58 (CGI-58) and inhibited by the G(0)/G(1) switch gene-2 protein (G0S2). G0S2 has also recently been identified as a positive regulator of oxidative phosphorylation within the mitochondria. Previous research has demonstrated in cell culture, a dose dependent mechanism for inhibition by G0S2 on ATGL. However our data is not consistent with this hypothesis. There was no change in G0S2 protein content during an acute lipolytic inducing set of contractions in both whole muscle, and isolated mitochondria yet both ATGL and G0S2 increase following endurance training, in spite of the fact that there should be increased reliance on intramuscular lipolysis. Therefore, inhibition of ATGL by G0S2 appears to be regulated through more complicated intracellular or post-translation regulation.

List of Abbreviations

AMP - adenosine monophsohphate
AMPK - adenosine monophsohphate activated protein kinase
ATGL – Adipose triglyceride lipase
ATP – Adenosine triphosphate
cAMP – Cyclic adenosine monophosphate
cDNA – Complementary deoxyribonucleic acid
CGI-58 – Comparative gene identification-58
DG – Diglyceride
FA – Fatty acid
G0S2 – G(0)/G(1) switch gene-2
HSL – Hormone sensitive lipase
IMTG – Intramuscular triglyceride
MG – Monoglyceride
MGL – Monoglyceride lipase
mRNA – Messenger ribonucleic acid
NEFA – Non-esterified fatty acid
NWA – Normal weight active
NWS – Normal weight sedentary
OBS – Obese sedentary
PKA – Protein Kinase A
PLIN1 – Perilipin 1
PPARγ - Peroxisome proliferator activated receptor γ
RG – Red gastrocnemius
SOL – Soleus
TG – Triglyceride
WG – White gastrocnemius

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Chapter 1

General introduction

Skeletal muscle insulin resistance is pivotal in the development of type II diabetes (72). The cause(s) of insulin resistance in skeletal muscle are currently unknown, but reports have linked higher intramyocellular lipids levels with the development of type II diabetes (29). Interestingly, two sub-populations of people have been identified as having a significantly increased intramyocellular lipid content, the obese and the endurance trained, colloquially known as the 'athletes paradox' because endurance trained individuals efficiently use fat as a fuel and are extremely insulin sensitive. This introduces the notion that intramyocellular content alone does not cause insulin resistance, however the dynamics between skeletal muscle anabolic and catabolic lipid processes appears to be a crucial physiological system worth further exploration (29).

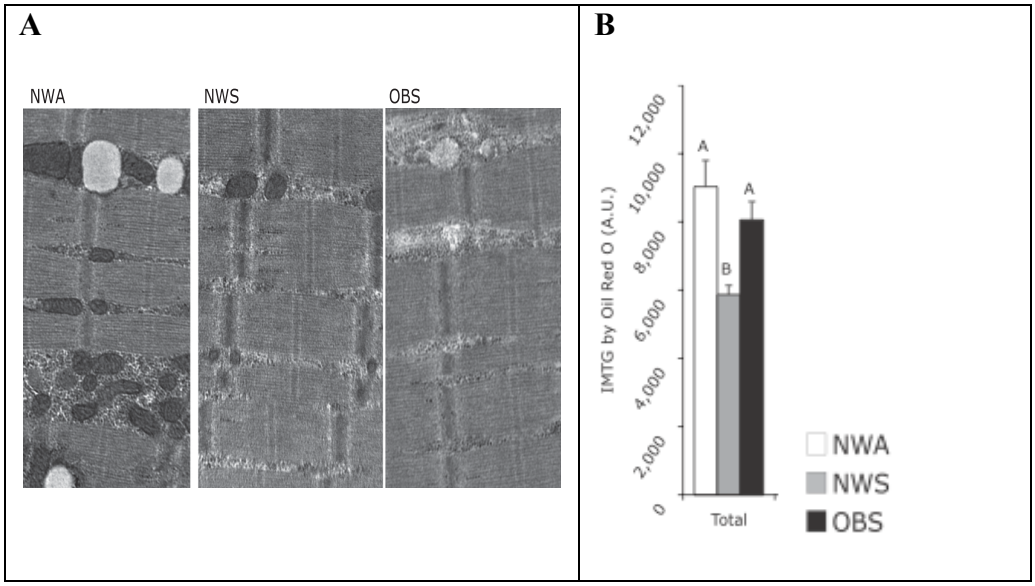

Figure 1. Adapted from Amati et al. (4).NWA: Normal weight active; NWS: Normal weight sedentary; OBS: Obese sedentary. **Figure A**: A cross sectional image of skeletal muscle. NWA has an organized large lipid droplet located next to the mitochondria, with OBS showing a scattered lipid formation compared to NWS. **Figure B:** Bar graph depicting IMTG content within skeletal muscle. NWA and OBS have elevated IMTG content when compared to NWS.

Improper regulation of lipolysis leads to incomplete breakdown products: fatty-acyl coenzyme A, diacylglyerols and ceramides that are thought to be lipotoxic. Therefore the

implications of the breakdown of stored lipids is imperative to healthy body functions (1, 4, 40). Intracellular lipids are stored within dynamic organelles known as lipid droplets. Research to date has been involved in the investigation of lipid droplet storage (synthesis) and use (lipolysis) within a variety of tissues, most notably adipose tissue. However, little work has been completed in skeletal muscle, and very little work has been done on the activation and inhibition of the rate-limiting lipase in skeletal muscle, specifically towards the potential inhibition of lipolysis.

This thesis examines the relationship of the rate-limiting enzyme, adipose triglyceride lipase (ATGL), to several protein regulators within a variety of known metabolically varying skeletal muscles, and the effects of endurance training on these proteins.

Chapter 2

Literature Review

Overview of lipolysis

Adipose tissue, specifically white adipose tissue, is the main energy storage site in mammals (50). Energy storage is defined as the difference between energy consumption compared to energy expenditure, and energy, when referring to mammals, comes in the form of food. If energy intake is greater than the body's energy demands, excess energy will then be stored in the form of adipose tissue. When non-esterified fatty acids (NEFAs) are consumed, or created through fatty acid biosynthesis from excess carbohydrates, they are esterified into an inert, non-active form known as triacylglycerol (TG) (50). The structure of a TG is three fatty acids bound to a glycerol backbone.

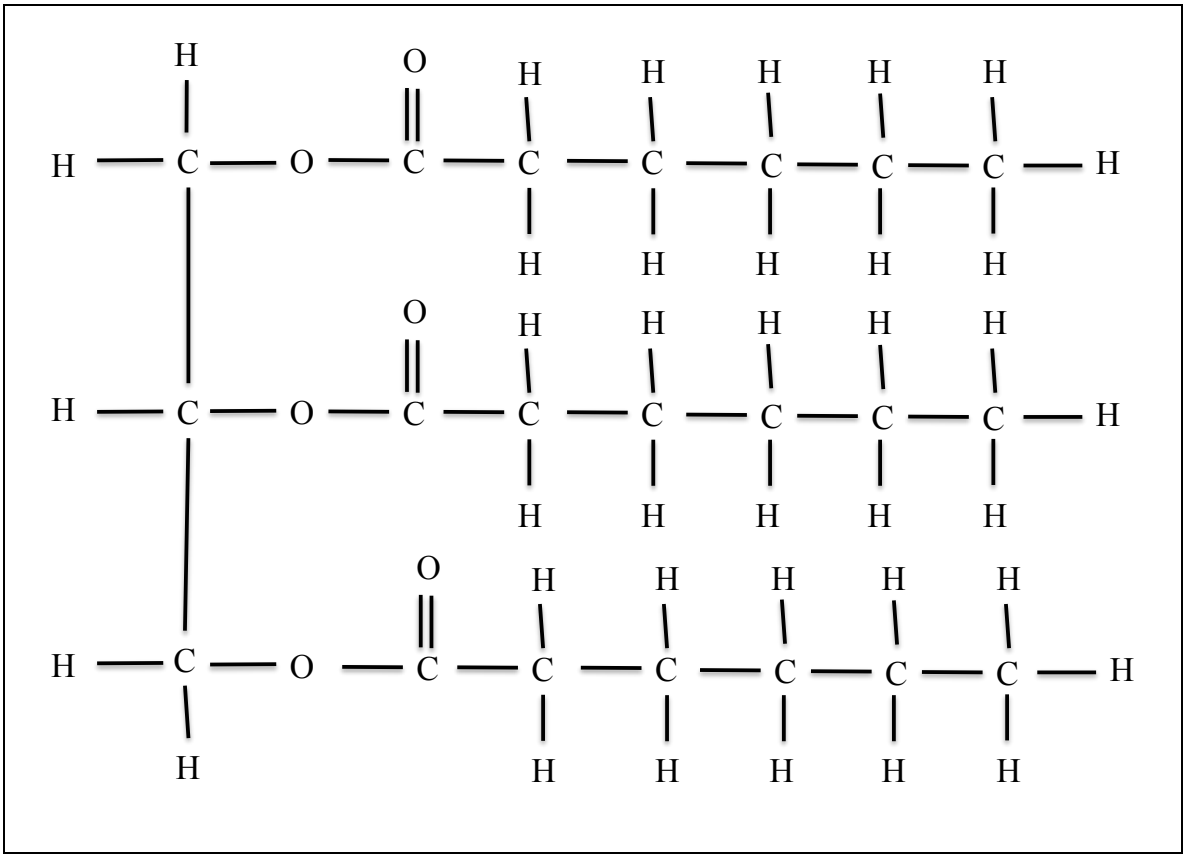

Figure 2. Basic structure of a triglyceride. With the removal of one of the three fatty acids, the compound is known as a diglyceride. With the removal of two of the three fatty acids, the compound is known as a monoglyceride.

In this form, a sequence of catalyzed reactions must ensue to release the fatty acid to be metabolized for ATP production. TGs are stored in organelles known as lipid droplets, and will remain until they are required to fuel ATP production. High levels of NEFAs are toxic to cell viability, and converting NEFAs into neutral TGs protects the cell from the cytotoxic effects (52, 79). When energy demands increase beyond energy intake, these lipid droplets will be catabolized starting with the hydrolysis of the TG and ending in the production of ATP through β-oxidation. This process of fatty acid release from TGs is known as lipolysis.

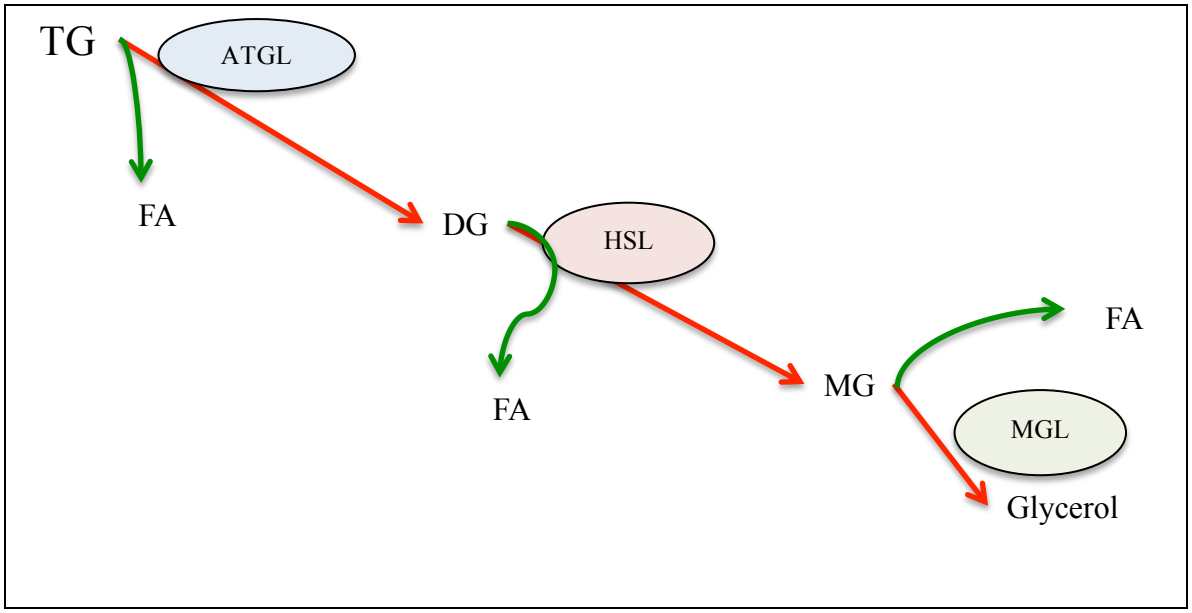

Figure 3. Visual representation of the lipolysis cascade. The removal of the first fatty (FA) acid from the triglyceride (TG) molecule is catalyzed by the first, and rate-limiting enzyme adipose triglyceride lipase. The removal of the second FA is catalyzed by hormone sensitive lipase (HSL), with the removal of the final FA being catalyzed by monoglyceride lipase (MGL).

Lipolysis begins with the hydrolysis of the first fatty acid, and the production of diglyceride (DG) through the activity of adipose triglyceride lipase (ATGL) (98). ATGL activity is under dual regulation and is the rate-limiting step in TG breakdown. It is activated by interaction with comparative gene identification-58 (CGI-58) (49), and

inhibited by the recently discovered G (0)/G (1) switch gene-2 (G0S2) protein (94). The complex interaction of ATGL and its two regulators, CGI-58 and G0S2, potentially dictate the rate at which lipolysis occurs. Once a DG is produced, hormone sensitive lipase (HSL) will catalyze the reaction of converting the DG into monoglyceride (MG) with the release of another fatty acid. The final fatty acid is cleaved off by monoglyceride lipase (MGL), releasing it from the glycerol backbone. The released fatty acids and glycerol from adipose tissue will be transported to, and used by other tissues depending on metabolic needs. Adipose tissue TG degradation is stimulated in times of increased whole body energy demand such as fasting or during exercise.

In adipose tissue, lipid droplets are surrounded by PLIN1, a protein only expressed in this tissue (53). PLIN1 is, in part, thought to allow for controlled access to TGs by degrading lipases (13). ATGL is found both on the lipid droplet and in the cytosol of adipocytes. While it is believed that ATGL is responsible for catalyzing basal lipolysis during times of low energy demand, it is not until activation by association with CGI-58 that ATGL is up regulated to increase fatty acid release (49). In a basal state, there is a constant cycling of TGs, with them being hydrolyzed into DGs, and then the cleaved fatty acids are re-esterified to reproduce TGs. In the basal state, CGI-58 is bound to PLIN1, but when G-mediated protein signaling is activated via adipocyte β-adrenergic receptors, intracellular cAMP levels increase, causing protein kinase A (PKA) activation (17). PKA then phosphorylates PLIN1 and HSL, causing HSL to translocate to the lipid droplet membrane (12). PLIN1 phosphorylation causes both the release of bound CGI-58, but also is thought to reconfigure the lipid droplet into smaller droplets allowing increased surface area for lipase exposure (13). During this process, cytosolic ATGL is

thought to translocate to the surface of the lipid droplet, and with increased interaction with the recently released CGI-58 from PLIN1, begins the lipolysis cascade (refer to Figure 4). Little is known about inhibition of these processes, but it is thought that G0S2 will inhibit not only ATGL activity, but also override any activation due to bound CGI-58, suggesting non-competing pathways for altering ATGL behavior (55). It is believed that ATGL is bound to G0S2 at all times; therefore further modification of either G0S2 or ATGL or both must ensue for a change in activation (94).

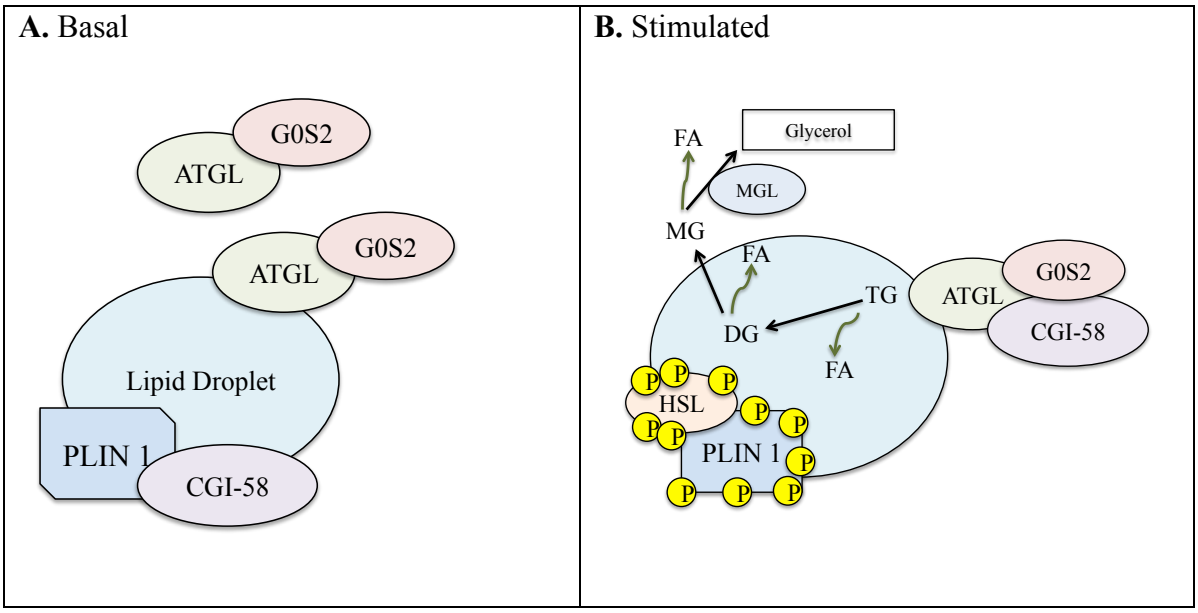

Figure 4. A. Depiction of basal adipose tissue state. **B.** Lipolysis is started by the co-activation of ATGL by CGI-58, followed by the phosphorylation of HSL, cause HSL to translocate to the lipid droplet, finally, MGL removing the final fatty acid leading to the complete hydrolysis of a TG for FA and glycerol release. Adapted from Yang et al. (94).

Lipolysis is not uniformly regulated across all lipid droplets in the body; there are several differences between adipose tissue and skeletal muscle lipolysis involving regulation of lipolytic pathways, and the actual lipid droplets themselves. Lipid droplets exist in many tissues such as liver, steroidogenic tissues and skeletal muscle. Skeletal

muscle TG breakdown is responsible for greater than 50% of the total fat breakdown during low to moderate intensity muscle contraction and exercise, but much less is understood about the regulation of muscle lipolysis (23, 60).

Lipolysis in skeletal muscle

Skeletal muscle is heavily reliant on fat oxidation for energy both at rest and during exercise. The mechanisms regulating lipolysis within skeletal muscle are still relatively unknown. However, it is thought that intramuscular TGs (IMTGs) are mobilized and consumed through metabolic (intracellular) and hormonal regulation. Unlike adipose tissue lipolysis, which is stimulated during times of whole body energy depletion, skeletal muscle lipolysis appears to be specific towards the individual energy demand of a given muscle, and is up regulated during exercise and down regulated in a state of insulin resistance due to a high fat diet (34, 44, 69). Whole body lipolysis is present even during periods of low exercise demand (e.g., at 25% VO_2max), however during increasing exercise intensities, use of plasma FA levels remained the same, yet IMTG lipolysis increases at higher intensities (65% VO_2max) and appears to be most efficient in this range (69). Fatty acids release from adipose tissue are systemically used for energy production in many tissues including skeletal muscle, whereas IMTG lipolysis is directed towards intracellular mitochondria for skeletal muscle energy production.

The relationship between ATGL and CGI-58 is better understood, especially in adipose tissue, however the interaction that the more recently discovered G0S2 has on ATGL (or possible CGI-58) is still relatively unknown. In adipose, CGI-58 is released with phosphorylation of PLIN1, which allows for the interaction between CGI-58 and

ATGL up regulating the activity of ATGL from a basal to active state, and in doing so, significantly increasing the rate of lipolysis (82, 92). Despite the apparent absence of PLIN1 in muscle (91), the interaction between CGI-58 and ATGL is still believed to be essential for the activation of ATGL to initiate the first cascading step of skeletal muscle lipolysis (22).

HSL was originally thought to be the only lipase responsible for complete hydrolysis of TGs in both adipose tissue and skeletal muscle (8), subsequent work using HSL-deficient mouse embryonic fibroblasts, TGs were still being catabolized, suggesting another non-HSL TG specific lipase (64). ATGL was only discovered as the rate-limiting lipase of TG hydrolysis in 2004 (98), and as such much more is known about the modulation of HSL in general and particularly within skeletal muscle.

Structure and Function of the Lipid Droplet

Although adipose tissue is the most abundant site of stored lipid droplets, the body can store a finite amount of lipid droplet in non-adipose tissue (22). There is only one lipid droplet existing within most adipocytes. The size of the lipid droplet can be ten times larger than lipid droplets found in non-adipocyte's at ectopic sites such as liver and skeletal muscle (83). While this may be the most prominent difference between adipocyte lipid droplets and non-adipocyte lipid droplets, many other differences do exist. In skeletal muscle, many small lipid droplets exist, and they are in proximity to both the mitochondria and the sarcoplasmic reticulum, mainly occupying the spaces between mitochondria in both the subsarcolemmal and myofibrillar regions (77). During times of increased energy turnover within skeletal muscle (i.e., exercise), it is believed that the

proximity of the lipid droplet to the mitochondria plays a functional role in accelerating fatty acid oxidation (84). The structures of most lipid droplets are very similar despite size differences. The basic structure of the lipid droplet comprises of a phospholipid monolayer surface with over 300 identified proteins embedded into the surface layer, and an esterified lipid core composed of TGs and cholesterol esters (62, 83). Lipid droplets are physiologically unique in that they are the only organelle without a phospholipid bilayer, and are surrounded by only a monolayer. Not much is known about lipid droplets as dynamic intracellular organelles, since they were originally thought to solely be inert lipid storage sites. It is postulated that the endoplasmic reticulum is the site of lipid droplet synthesis (11).

Adipose Triglyceride Lipase

The first fatty acid of the TG was originally thought to be cleaved off solely by HSL which has both TG and DG lipase activity (8). It was hypothesized that changes in the phosphorylation state of HSL by cAMP-mediated PKA activity dictated its catalytic behavior, and through this, altered the rates of lipolysis (8). While it has been proven that HSL can indeed cleave off a FA from a TG, it has a ten-fold greater affinity for DGs over TGs (25). However, in HSL knockout mice, DG accumulation in adipose tissue suggested that another lipase was responsible for the rate-limiting step of lipolysis since TGs were being catabolized into DGs even in the absence of HSL, contrary to previous belief (32). This was observed across several tissues, demonstrating that whatever this preferential lipase towards TGs was, it was not adipose tissue specific, due to DG accumulation in skeletal muscle and liver as well as adipose tissue (32). In 2004,

Zimmerman et al. identified through gene and protein screening, a protein that matched other lipases, yet had the ability to catalyze the reaction of TG to DG while releasing a fatty acid. This was further proven with the demonstration that adipose triglyceride lipase (ATGL) causes FA release from TGs using ATGL extracts *in vitro* (42, 87, 98). At the time of discovery, two other groups identified desnutrin (87) and calcium-independent phospholipase A2ζ (42) that are now known to be identical proteins to ATGL. The role of ATGL is best understood in adipose tissue, but it is currently not as well understood in skeletal muscle. In an ATGL knockdown mouse model, there was a significant decrease in TG catabolism and lipolytic activity causing a shift to glucose metabolism (31). Subsequently, in the ATGL-knockout mouse, TG accumulation in cardiac tissue caused premature death due to cardiomyopathy (31). Schoiswohl et al. (74) developed an ATGL-knockout mouse without the ability to produce ATGL in all tissues except the heart (cardiac muscle specific ATGL-knockout). The cardiac muscle specific ATGL-knockout mouse had a normal life span, but was unable to increase FA levels during exercise leading to a rapid depletion of liver glycogen, suggesting ATGL as a crucial regulator of energy control in skeletal muscle during times of heightened energy demand (74). ATGL also has the capability for transacylase activity, although the purpose of this function of this is currently unknown (42)

ATGL, unlike HSL, has a strong substrate affinity towards TGs, with very low catabolic ability towards DGs (98). Like HSL, ATGL expression appears to be predominantly in oxidative type I muscle fibers (43). To date, we have an incomplete understanding of the regulation of ATGL, particularly in skeletal muscle. Regulation of this lipase appears to be crucial in the rates of lipolysis across all tissues.

Hormone Sensitive Lipase (HSL)

Not only was it thought that HSL was the lipase responsible for both the initial step in lipolysis, the breakdown of TGs into DGs, but also the subsequent step of hydrolyzing DGs into MGs (25). The original belief was that HSL was the sole lipase responsible for the catalyzing the breakdown of TGs into FAs for specific metabolic fates. *In vitro* experiments also demonstrate that HSL can hydrolyze MGs, but to a much lesser extent (25). A non-obese mouse HSL knock-out was produced and demonstrated an accumulation of DGs within their adipose tissue, it lead to the the realization that another enzyme responsible for the first reaction must exist, but also indicated that the primary role of HSL is most likely to catalyze DGs into MGs (32, 64, 88, 97). HSL is most abundantly expressed in white and brown adipose tissue, but also expressed in other tissues such as smooth, cardiac and skeletal muscle (35, 46). HSL activity is regulated through phosphorylation by both protein kinase A (PKA) and adenosine monophsohphate (AMP) – activated protein kinase (AMPK) (5, 27). During basal state, HSL is phosphorylated at serine (Ser-)565 by AMPK to block phosphorylation by PKA converting HSL into an antilipolytic state (27, 28). *In vitro* experimentation of rat adipocytes led to the understanding that HSL has several phosphorylation sites and is involved in complex regulation with potentially Ser-659 and Ser-660 being the two most important sites for HSL up-regulation by PKA activation (5). PKA phosphorylation of HSL via β-adrenergic stimulation accounts for a 2-fold increase in HSL activity, yet β-adrenergic activation of PKA causes a 100-fold increase of TG hydrolysis (96). The

regulation of HSL is important in the process of lipolysis, however it is no longer thought to be the sole lipase responsible for the breakdown of TGs.

Monoglyceride Lipase (MGL)

MGL is currently recognized as the enzyme responsible for MG hydrolysis, catalyzing the reaction of the removal of the final fatty acid from the glycerol backbone and releasing both the fatty acid and the glycerol for their respective roles in metabolic processes. Activity of MGL is roughly ten-fold higher than that of ATGL, suggesting that MGL is not rate limiting in terms of overall lipolytic rates and its activity is substrate driven (18). To study this further, Taschler et al. (85) developed an MGL knockout mouse. The MGL knockout mice had a significant decrease in MG hydrolase activity, and an increase of MG across a variety of tissues, indicating that it is important for this final step.

Comparative Gene Identification-58 (CGI-58)

In the search for novel human genes, *Caenorhabditis elegans* was used as a scaffold for comparative identification of human genes. The complete genome composition was determined for C*aenorhabditis elegans,* a roundworm, therefore using the proteome from this, the first completed genome of a multicellular organism allowed proper identification of human like genes. The nomenclature was given as CGI, comparative gene identification. Over 150 full-length human genes were identified (47). Several mutations were identified in one of the CGI genes as being related to Chanarin-Dorfman Syndrome, a syndrome related to the manifestation of high concentrations of

intracellular TGs across many tissues (15). Eight mutations were discovered in the CGI-58 gene region in patients with Chanarin-Dorfman Syndrome (51). While it was noted that CGI-58 was not a lipase, it was discovered that CGI-58 had an activating effect on lipolysis, as it was established that it positively regulates ATGL hydrolysis by an increase of greater than 20-fold (49).

CGI-58 up regulates ATGL activity

ATGL is active under basal conditions, however, when activated by CGI-58, TG hydrolysis is increased 20-fold (49). In the absence of ATGL, CGI-58 cannot increase lipolytic activity via any other route; therefore it is dependent on ATGL to influence lipolytic activity (75). To increase ATGL activity, a physical interaction must exist between ATGL and CGI-58, although details on this interaction are not well understood. In cultured adipocytes from CGI-58 knockout mice, Osumi and colleagues (93) were able to demonstrate that, in both basal and PKA stimulated lipolysis, TG hydrolysis rates did not change in the absence of the co-activator, demonstrating the importance of CGI-58 on lipolytic rates. In 3T3-L1 adipoctyes, CGI-58 knockdown induced a 20% decrease in lipolysis in the basal condition; suggesting a role for CGI-58 even in a basal state.

In adipose tissue, it is believed that PLIN1 releases CGI-58 into the cytosol to increase the activity of ATGL through a physical interaction (82, 92). Although PLIN1 is absent in skeletal muscle (91), the interaction between CGI-58 and ATGL still appears to be important in controlling lipolytic rates (22). During muscle contraction, ATGL activity is significantly increased by interaction with CGI-58, demonstrating that another mechanism for lipid turnover exists outside of adipose tissue (58). While the regulation of

ATGL is currently not well understood in skeletal muscle, the interaction between ATGL and CGI-58 appears to be pivitol in the activation of lipolysis. In skeletal muscle lysates, the progressive addition of CGI-58 led to a dose dependant increase of TG hydrolysis (74). Alternatively, in ATGL deficient adipose tissue in culture, the introduction of CGI-58 alone was unable to induce lipolysis, suggesting that CGI-58 only upregulates ATGL as a target for modulating lipolysis (75). However, the specific mechanism by which CGI-58 interacts with ATGL is still unknown. In HeLa cells co-expressed with ATGL and CGI-58, it appears that CGI-58 is always located around the lipid droplet, whereas ATGL is found both on and off the lipid droplet, it is believed that ATGL translocates to the lipid droplet anchored by its' hydrophobic domain (55). Removal of the hydrophobic domain of ATGL prevented ATGL from anchoring to the lipid droplet, causing excess ATGL in the cytosol, however, with CGI-58 co-expression ATGL was able to move towards the lipid droplet starting lipolysis.

G(0)/G(1) Switch Gene-2 protein

The G0/G1 switch genes (G0S) were identified using cDNA libraries to examine oncogenes that may have an impact in lymphotropic viruses and are involved in inducing cells to go from the G0 to the G1 phase in the cell cycle (80). In cultured blood mononuclear cells, the G0/G1 switch gene-2 (G0S2) protein was discovered during the G0 to G1 phase of a drug-induced cell cycle (71). G0S2 is derived from a 103 amino acid sequence with the gene found on chromosome 1 (71, 80). Protein structure analyses have led to the discovery of two potential phosphorylation sites from protein kinase C and casein kinase II (71). However, no evidence yet exists as to whether G0S2 can be

phosphorylated under physiological conditions. Implications have been made about functions of G0S2, it is involved in cell proliferation (80), apoptosis, inflammation, carcinogenesis, but the most prominent function appears to be in down regulation of ATGL activity (55, 94). G0S2 expression is much higher in brown and white adipose tissue, but is still found in other metabolically active tissues such as the liver, heart and skeletal muscle (2, 95).

In G0S2 overexpressing HeLa cells, G0S2 localizes to the lipid droplet membrane and prevents TG hydrolysis (94). Whereas in a G0S2 knockdown HeLa cell, basal and stimulated lipolysis were both significantly increased (94). Taken together this adds further evidence that the mechanisms involved in actual the interaction between G0S2 and ATGL still remain unknown, however it appears that G0S2 and ATGL are bound regardless of ATGL activity and even in the presence of CGI-58 (55). In a G0S2 overexpressing cell line, activation of ATGL through CGI-58 was unable to increase lipid droplet degradation, suggesting non-competing pathways for lipid droplet control (55). It is suggested that the potential inhibition of ATGL by G0S2 is done through G0S2 blocking substrate availability to ATGL, and that G0S2 and CGI-58 act on ATGL through separate non-competing mechanisms since regardless of CGI-58 presence and interaction with ATGL, G0S2 will dose dependently inhibit ATGL function (55).

Not only is G0S2 important in the down-regulation of ATGL, it also may play a functional role in adipogenesis as well. Lee et al. (2) examined both the fat cell fraction and the stromal vascular fraction of adipose tissue. The fat cell fraction contains adipocytes, while the stromal vascular fraction contains mostly undifferentiated pre-adipocytes. The expression of G0S2 was over 16-fold higher expression in the fat cell

fraction compared to the stromal vascular fraction, suggesting a possible role in adipogenesis and lipid accumulation during adipocyte development. During 3T3-L1 adipogenesis, G0S2 increased significantly during the adipogenesis cycle (95). This is consistent with G0S2 being up regulated by the transcription factor: peroxisome proliferator activated receptor γ (PPARγ) (95). PPARγ is highly expressed in adipose tissue, but is also present in skeletal muscle, and is viewed as an overall regulator of adipogenesis (6, 68, 70, 86, 95). The G0S2 gene also contains a carbohydrate response element resulting in direct sensitivity to altered glucose concentrations (56). Parikh et al. (65) in skeletal muscle following a hyperinsulinemic euglycemic clamp (*in vivo*) discovered that G0S2 mRNA expression rose significantly demonstrating the acute responsiveness to elevated insulin concentrations and increased muscle glucose uptake. In human adipose tissue following fasting conditions, an increase in ATGL mRNA and protein expression was observed, yet a decrease in G0S2 mRNA and protein expression is observed (63). This is understandable since in a fasting state, there is a heightened need for fat mobilization through increased lipase activity and a decreased need for inhibition of lipolysis. This is also consistent with the potential inhibiting mechanism of G0S2 being dose dependently regulated, since a decrease in G0S2 would facilitate ATGL catalytic ability. However, these data challenge the notion that G0S2 and ATGL are coordinately regulated, and obviously not bound to a one-to-one stoichiometry in adipose tissue. Therefore, further exploration into the contribution of G0S2 inhibition to the regulation of lipolysis is warranted. Endurance training caused increases in ATGL protein content, with no changes in CGI-58 and no changes in total G0S2 protein content in vastus lateralis muscle biopsies from obese adult men (54). However, this does

corroborate the idea that G0S2 is a dose dependent modulator since relative to ATGL content, G0S2 protein did decrease.

Recently, two separate studies have identified G0S2 as a specific mitochondrial protein, albeit for almost contradictory purposes. Kioka et al. (45) recently identified G0S2 as a positive regulator of oxidative phosphorylation via up regulation of complex V (F_0F_1 ATPase). On the other hand, Welch et al. (90) identified G0S2 as a pro-apoptotic protein, interacting with Bcl-2 to prevent the anti-apoptotic heterodimer formation with Bcl-2/Bax. When exposed to cold temperatures, G0S2 knockout mice were better adapted to the temperature changes by up regulating known thermogenic genes, potentially indicating another mitochondrial role for heat production (57). Given this evidence that G0S2 is possibly not only a dose dependent inhibitor of ATGL, but a pro-apoptotic protein and a positive regulator of oxidative phosphorylation; more research is needed to ascertain a true function of G0S2 for energy regulation.

Skeletal muscle fiber type and the effects of endurance training on IMTG metabolism

Skeletal muscle fiber types can be separated into three general classifications based on several techniques such as histochemical analysis, mitochondrial enzyme content and ATPase activity (7, 14, 19, 66). In broad terms, skeletal muscle fiber types can be divided into type I, or slow twitch fibers, and type II, or fast twitch fibers. Recently, seven skeletal muscle fiber types have been recognized, but for the purpose of this review only three general classifications will be examined type I, type IIa and type

IIb/x, since these are three broad classifications that can relate to rat skeletal muscle tissue.

Fiber type classification		
mATPase	**Myosin heavy chain**	**biochemical**
I	**MHCI**	**SO**
IC		
IIC		
IIAC		
IIA	**MHCIIa**	**FOG**
IIAB		
IIB	**MCHIIx/d(IIb)**	**FG**

Table 1: Table of fiber type classifications with specifics on type I, IIA and IIB fibers. Adapted from Scott et al. (76)

Type I fibers have slower contractile properties (i.e., time to peak tension, $t_{1/2}$ relaxation time), yet longer time to fatigue. They rely more heavily on fat oxidation for fuel, which contributes to their greater fatigue resistance. Type IIb/x have the fastest contractile properties, yet fatigue relatively quickly when compared to type I and IIa, relying more on anaerobic and glycolytic energy pathways. Intermediate type IIa fibers generally are in between the contractile properties compared to type I and IIb/x, but have high oxidative capacity and high glycolytic capacity as well (reviewed in (76)). Both type I and type II fibers store IMTGs, although type I has a greater IMTG content. Both fiber populations demonstrate decreased IMTG content (i.e., IMTG utilization) following an acute bout of endurance exercise (78). The rat model allows us to select muscles that are predominantly fiber type specific, unlike human skeletal muscle where even more

oxidative tissues are mostly mixed fibers. The rat soleus muscle is predominantly a postural muscle and contains a high percentage of type I fibers (84% type I; 7% type IIa and 9% type IIb/x) (19). The gastrocnemius muscle can be excised from both the medial and lateral portions, yielding both a 'red' and a 'white' sample. The red gastrocnemius is considered largely type IIa (51% type I; 35% type IIa and 14% type IIb/x) and is highly oxidative and glycolytic, and the white gastrocnemius is highly specialized fast-twitch glycolytic fibers (0% type I; 0% type IIa and 100% type IIb/x) (19).

Following a single exhaustive swimming bout, a decrease in IMTG content was noted amongst a variety of rat skeletal muscles. Specifically, a reduction of 48% of IMTG in type I soleus, a reduction of 29% in red gastrocnemius (predominantly type IIa), with no noticeable difference in IMTG content in the type IIb white gastrocnemius, demonstrating preferential depletion of IMTG within oxidative skeletal muscle fiber types (81). In skeletal muscle from obese rats following a hyperinsulinemic-euglycemic clamp, a reciprocal response was observed between the soleus when compared to mixed gastrocnemius, in that there was an increase in IMTG turnover by over 3-fold within the soleus, yet decreased IMTG turnover in the gastrocnemius. Prior to the clamp the gastrocnemius had a higher IMTG fractional turnover rate compared to the soleus, yet following the clamp it was reversed (30). This demonstrates that alterations in IMTG turnover are not the same in all fiber types, and that the study of a variety of skeletal muscle types is warranted.

Following prolonged endurance training, the fiber types are altered with noted increases in type IIa fibers and decreases of type IIb/x fibers, with little or no change in oxidative capacity of the type I fibers (16, 41, 59). However, there is a greater increase in

mitochondrial content within type II fibers such that after chronic endurance training there is little to no difference in mitochondrial content between type I and type II, indicating a shift in fiber type largely through increased mitochondrial biogenesis (16, 41, 59). They also discovered, however, no increase in β-adrenergic receptor density (59). This is important to note since β-adrenergic receptor activation by catecholamines promotes lipolysis in a variety of tissues, and it has been shown that mutations in a variety of β-adrenergic receptor codons can cause obesity (albeit in adipose tissue) (48). Similarly, beneficial mutations in β-adrenergic receptor codons have been known to cause an increase in aerobic capacity (73). With a decrease in catecholamine stimulation, and no increase in β-adrenergic receptor density, increased reliance on IMTG lipolysis must be regulated by other, currently unknown pathways (37).

IMTG dynamics and endurance training

In skeletal muscle from sedentary mammals, IMTG pools only account for roughly 1% of whole body lipid storage. However, during endurance exercise, skeletal muscle lipolysis accounts for greater than 50% of fat oxidation with the remainder presumably from oxidation of plasma free fatty acids (60). Whole body reliance on plasma free fatty acids increases, and a significant reduction in rates of glycogen utilization are observed both during and after endurance training. A central adaptation to endurance training in terms of skeletal muscle is that there is an increase in carbohydrate sparing, with an increase in reliance upon fat oxidation for fuel provision (24, 33, 34). Low-to-moderate chronic exercise is a powerful inducer of mitochondrial biogenesis of between 50-100% increase within 6 weeks (reviewed by (36)). There is also an increase

in lipid droplet-to-mitochondria association following endurance training (4, 20). Training results not only in an increase in IMTG utilization, but significant muscle glycogen sparing as well. Hurley et al. (39) observed that following 12 weeks of moderate intensity endurance training in humans, there was a significant decrease in respiratory exchange ratio, but also in a single bout of exercise 48 hours after the training protocol there was a decrease in plasma free fatty acids and glycerol, indicating an increased reliance on endogenous (or intramuscular) fat stores to sustain energy demands during exercise. There was also a 41% increase in muscle glycogen sparing, further indicating IMTG content as being relied upon as energy stores, which was corroborated further by increased storage at rest and a greater decrease in IMTG content during the single bout of exercise following endurance training compared to before. Changes in intramuscular shifts towards fat oxidation can be observed as quickly as 31 days, as noted by Phillips et al. (67), who noticed increased IMTG depletion following exercise as quickly as 5 days into a chronic training regime, with more substantial changes at 31 days.

Controversy exists throughout the literature concerning the utilization of IMTG before and after endurance training due to the variability in measurements of IMTG content (reviewed by (89)). However, there is some evidence that there is a greater reliance on IMTG for energy production following endurance training. In elite endurance trained cross-country skiers following a roughly 420 minute race there was a decrease in IMTG content by 36.4 mmol/kg dry mass compared to pre race suggesting that IMTGs are an important fuel during strenuous prolonged exercise in elite endurance athletes (10, 26). In a longitudinal study, there was significant increase in IMTG utilization during a

single bout of moderate intensity exercise in subjects who participated in twelve weeks of endurance training compared to their untrained state (38).

Endurance training is also known to increase insulin sensitivity. There has been some evidence that this may be related to changes in intramuscular DG and ceramide levels, which are potentially lipotoxic, and accumulation has been linked to insulin resistance (1, 40). In older obese men and women, muscle DGs and ceramide content decreased following 16 weeks of endurance training, with corresponding decreases in body weight and fat mass (21). However, in a similar endurance training study with lean and obese women, body weight did not change and neither did DG or ceramide content, suggesting that adiposity may contribute to lipotoxicity regardless of training status (20).

With endurance exercise there is also an increase in IMTG synthesis and storage suggesting that the dynamic turnover of IMTGs is important in the increase in IMTG utilization (9, 61). However, during tetanic stimulation of rat soleus following endurance training, Dyck et al. (24) demonstrated that while there was an increase IMTG synthesis during contraction (endurance exercised vs. control), IMTG hydrolysis and oxidation were reduced in the exercised group by 49% and 30% respectively. This was the first study to analyze IMTG turnover in the soleus alone, and the results are surprising given that several other studies have indicated increased IMTG utilization following endurance training. However, the soleus is a very unusual muscle given its postural role and high type I fiber content, and therefore it would be important to study fiber type differences in IMTG utilization adaptations to endurance exercise.

In human skeletal muscle following endurance training, ATGL protein content increases, which would be consistent with higher rates of IMTG breakdown (3, 4). Alsted

et al. (3) discovered that, while ATGL is higher in skeletal muscle in endurance-trained men compared to sedentary controls, neither HSL nor CGI-58 content was higher. In spite of this, basal lipolysis increased, suggesting that any increase in IMTG hydrolysis is mediated by ATGL. Interestingly, even though they observed no change in HSL content, there was no increase in DG accumulation - suggesting that HSL activity is not rate limiting and must be in excess under both trained and untrained conditions. It would also appear that since CGI-58 content remained unchanged, it is potentially saturated in skeletal muscle, and that its positive regulatory effects on ATGL are driven solely on the changes in concentration/activation of ATGL. Little is known in skeletal muscle regarding the regulatory protein G0S2, and no study to date has examined G0S2 expression following endurance training in healthy tissue.

Chapter 3

Statement of the problem

In the past ten years, ATGL has been recognized as the rate limiting lipase responsible for commencing the cascade of lipolysis in adipose tissue and skeletal muscle. Most studies examined only ATGL protein and mRNA expression with different metabolic perturbations, but more recently studies have been including CGI-58 co-expression as an outcome measure, with most research focusing only in cell models or adipose tissue. Very little research to date has focused on co-ordinate expression of ATGL, CGI-58 and G0S2 across non-adipose tissues, as well as combining all three under one perturbation. Research to date has demonstrated that expression of ATGL and G0S2 is not similar in response to fasting in adipose tissue, dispelling the notion that ATGL and G0S2 are always co-expressed, further adding evidence to the potential mechanism of G0S2 inhibition on ATGL in a dose-dependent manner.

Purpose and hypotheses

The purpose of this thesis is two fold: 1) To determine relative protein content of ATGL, CGI-58 and G0S2 across three metabolically diverse skeletal muscles in order to give insight into the relationship between ATGL, CGI-58 and G0S2 and perhaps the dose-dependent inhibition of G0S2 on ATGL. 2) To determine the effects of endurance training on ATGL, CGI-58 and G0S2 across a variety of skeletal muscles to determine fiber type differences, if any, on protein content, as well as to determine if during an acute lipolytic inducing contraction whether changes in G0S2 mirror that of IMTG breakdown.

We hypothesize that the soleus will have the greatest ATGL protein content compared to the red and white gastrocnemius, with the inverse for G0S2 protein content

across the same tissues, with the soleus having the least amount of inhibitor G0S2 relative to ATGL protein and the highly glycolytic white gastrocnemius having the highest amount of G0S2 relative to ATGL. Following endurance training, we expected to see increases in ATGL in all three of our skeletal muscles, with no changes in CGI-58, and decreased inhibitory G0S2 with the greatest decreases in the oxidative soleus muscle.

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Chapter 4

(Formatted for submission to PLOSone)

Division of labor:

Patrick C. Turnbull: Involved in experimental design, conducting all experiments and biochemical analysis, troubleshooting methodology and pioneering analysis of G0S2 protein and mRNA analysis for the lab, writing and editing of manuscript
Sofhia V. Ramos: Helped with conducting western blot experiments for troubleshooting
Rebecca E. K. MacPherson: Involved in experimental designs, writing and editing of manuscript
Brian D. Roy: Involved in experimental designs, writing and editing of manuscript
Sandra J. Peters: Academic supervisor, involved in experimental designs, troubleshooting methodology, writing and editing of manuscript.

Characterization of lipolytic inhibitor G(0)/G(1) switch gene-2 protein (G0S2) expression in skeletal muscle and heart compared to relative content of adipose triglyceride lipase (ATGL) and comparitive gene identification-58 (CGI-58).

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ABSTRACT

The rate-limiting enzyme in lipolysis, ATGL, is co-activated by comparative gene identification-58 (CGI-58) and inhibited by the G(0)/G(1) switch gene-2 (G0S2) protein. There is little work examining G0S2 expression in lipolytic tissues, and the relative expression across tissues or in skeletal muscle has not yet been described. Three muscles, soleus (SOL), red gastrocnemius (RG), and white gastrocnemius (WG), as well as heart were excised from 57-day old male Sprague-Dawley rats (n = 9). QRT-PCR was used for mRNA analysis, and western blotting was conducted to determine protein content. ATGL and G0S2 protein content were both greatest in the lipolytic SOL, with the least amount of both protein content found in the WG. CGI-58 protein content however did not mirror ATGL and G0S2 protein content, since the RG had the greatest protein content when compared to the SOL and WG.

When comparing our tissues based on CGI-58-to-ATGL ratio and G0S2-to-ATGL ratio, it was discovered that contrary to oxidative demand, the glycolytic WG had the greatest co-activator CGI-58-to-ATGL ratio with the oxidative SOL having the least, and no differences in G0S2-to-ATGL across the three muscle types. These data suggest that the content of G0S2 relative to the lipase in skeletal muscle would not predict lipolytic potential.

INTRODUCTION

Adipose triglyceride lipase (ATGL) has been described as the rate-limiting enzyme of lipolysis (25). ATGL is responsible for catalyzing the removal of the first fatty acid from the glycerol backbone of triglycerides (TG), releasing fatty acids for energy production and subsequently producing diglycerides (25). It has recently been demonstrated that ATGL has dual regulation, where it is activated by comparative gene identification-58 (CGI-58) and inhibited by the G(0)/G(1) switch gene-2 protein (G0S2) (10, 14, 23). Most studies to date have been conducted in cell culture or in adipose tissue, but relatively little is known about the relationship of these three proteins in other metabolically active tissues, such as skeletal muscle.

It appears that ATGL activity is directly influenced by both CGI-58 and G0S2 since the presence or abundance of these proteins has been demonstrated to alter the hydrolyzing rate of ATGL (10, 14, 19), with either activation or inhibition, respectively. Yet nothing is known of the ATGL-to-G0S2 or ATGL-to-CGI-58 content ratio amongst non-adipose lipolytic tissues such as skeletal muscle and heart. In G0S2 overexpressing HeLa cells, G0S2 localizes to the lipid droplet membrane and prevents TG hydrolysis (23). In the same HeLa cell model the loss of G0S2 by knockdown expression caused both basal and stimulated lipolysis to be significantly increased (23). This would suggest that at least in cultured HeLa cells, the content of G0S2 could affect the rate of lipolysis. In skeletal muscle, Parikh et al. (17), demonstrated that G0S2 mRNA expression rose significantly following a hyperinsulinemic euglycemic clamp (*in vivo*), demonstrating the acute responsiveness of the G0S2 gene to elevated insulin concentrations and increased muscle glucose uptake. This would indicate that G0S2 protein content might be expected

to decrease lipolysis in response to insulin, although protein content was not measured in this study. Taken together, these data would suggest that the relative content of G0S2 to the ATGL lipase could play an important role in regulating lipolysis. Although the mechanisms behind the regulation of ATGL by G0S2 are unknown, it appears that G0S2 and ATGL are bound together regardless of ATGL activity and lipolysis can be down-regulated even in the presence of CGI-58 (14). In human adipose tissue following fasting, an increase in adipose ATGL mRNA and protein expression was observed, yet a decrease in G0S2 mRNA and protein expression was also observed (15). This is understandable since in a fasting state, there is a heightened need for fat mobilization through increased adipose ATGL lipase activity and a decreased need for inhibition of lipolysis. These data suggest that expression of the lipase and G0S2 may play a role in regulating lipolytic rates, at least in adipose tissue. This inverse response in protein content also challenges the notion that G0S2 and ATGL are coordinately regulated, and obviously not bound to a one-to-one stoichiometry in adipose tissue. However, little is known about G0S2 in other tissues (e.g., skeletal and cardiac muscle) and therefore, further exploration into the contribution of G0S2 inhibition to the regulation of lipolysis is warranted. With tissue-specific differences in metabolic demands, perhaps the relationship between ATGL, CGI-58 and G0S2 can be indicative of known lipolytic profiles across a variety of lipolytic tissues.

However, G0S2 does appear to play an alternate role within mitochondria (9, 22). In cultured ATP depleted hypoxic cardiomyocytes, G0S2 interacts with Complex V (F_OF_1-ATP synthase) positively regulating ATP production (9). Interestingly, in human primary fibroblasts placed under TNF-α stress, G0S2 binds to Bcl-2, preventing the Bcl-

2/Bax anti-apoptotic heterodimer, thereby promoting apoptosis (22). While the functions appear to vary, it is interesting to note specific functions of G0S2 within mitochondria as a cell modulator in not only energy production, but also cell viability.

Most studies to date have focused on G0S2 mRNA expression, and few studies have reported G0S2 protein content. Even fewer studies have examined G0S2 in skeletal muscle, so there is little understanding of the basal protein content across varying oxidative muscle tissues (13, 17).

The purpose of this study was twofold: 1) to determine ATGL, CGI-58 and G0S2 mRNA expression and protein content in three metabolically different rat skeletal muscles (i.e., soleus, red gastrocnemius and white gastrocnemius), as well as in heart; and 2) to determine the G0S2-to-ATGL and CGI-58-to-ATGL ratios within the skeletal muscle tissues to gain information on whether the relative content in these tissues would correspond to the relative reliance on lipolysis for energy production.

METHODS

Animals

Male Sprague-Dawley (n = 9; age 56 days, body mass 272 ± 2 g), were used for this study. Rats were ordered from Charles River Laboratories (Quebec, Canada). Animals were housed for 48 hours after delivery in paired cages in a controlled 12:12 hour light to dark cycle in the Brock University Animal Facility. Food was fed ad libitum with access to water at all times. All experimental procedures and protocols are approved by the Brock University Animal Care and Utilization Committee and conformed to all Canadian Council on Animal Care guidelines (Animal Utilization Project Protocol permit number: 13-03-03)(16).

G0S2 Knockout mice

Mixed quadriceps muscle (n = 2) of both wild type (WT) and global G0S2 knockout (KO) mice were generously provided by Dr. Jun Liu from the Mayo Clinic in Arizona (24)

Muscle extraction

Rats were anaesthetized by intraperitoneal injections of sodium pentobarbital (6 mg/100 g body wt). The soleus (SOL), red gastrocnemius (RG), white gastrocnemius (WG) and heart were all removed, immediately snap frozen in liquid nitrogen, and stored at -80^{o}C until analysis.

Citrate Synthase

The SOL, RG, WG and heart were assayed for citrate synthase activity, as previously described by our lab (11). Briefly, frozen tissue was homogenized in 1 M K_2HPO_4 buffer

at pH = 8.1. In a cuvette, a real time reaction was initiated by adding the substrates oxaloacetate, triton, acetyl CoA into either tissue homogenate or a blank. Subsequently produced free CoASH reacted with 5,5′-dithiobis-(2-nitrobenzoic acid) for colorimetric analysis on a GE Ultrospec 2100 pro spectrophotometer (Baie d'Urfe, Quebec, Canada) at 412 nm (21).

mRNA extraction

mRNA was extracted using a Trizol method (Invitrogen, Carlsbad, USA). Complementary DNA (cDNA) was reverse transcribed from total RNA using a High Capacity RNA-to-cDNA Kit (Invitrogen, Carlsbad, USA). After reverse transcription, cDNA was kept at -20^{o}C.

Real Time PCR

SYBR green fluorescent Master Mix was used in all reactions (Invitrogen, Carlsbad, USA) with Acidic Ribosomal Protein 36B4 (36B4) as our endogenous house keeping control gene (2). Primers were hand selected for ATGL, CGI-58 and G0S2 (Table 1, Integrated DNA Technologies). Real time PCR was carried out using a Step One Plus thermal cycler (Applied Biosystems). Differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method (12).

Table 2. Name and sequences of primers

Primer name	Forward sequence	Reverse sequence
ATGL	5'- AGA CTG TCT GAG CAG GTG GA – 3'	5' – AGT AGC TGA CGC TGG CAT TC – 3'

CGI-58	5’ – TAC CCG TCA AGG GTC AGT CA – 3’	5’ – CAG CAA GAT CTG GTC GCT CA – 3’
G0S2	5’ – AGC ATG CCT CTT AAG GCT GG – 3’	5’ – GGA TTC GGT GGC ACC TTG AA – 3’
36B4	5’ – ATG TGC AGC TGA TAA AGA CTG GA – 3’	5’ – TGA TCA GCC CGA AGG AGA AG – 3’

Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 8% (for CGI-58), 10% (for ATGL) or 15% (for G0S2) running gel, all with 4% stacking gel. Separation was performed at 120V for 90 minutes and transferred onto either a 0.20μm or 0.45μm polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) using a 100V current for 1 hour. Blocking was done in either 2% fat free milk in Tris Buffered Saline (TBST) (for CGI-58), 5% fat free milk in TBST (for G0S2), or in 5% bovine serum albumin (BSA) in TBST (for ATGL). Anti-ATGL primary antibody (Cell Signaling Technology, Beverly, Massachusetts, USA #2439s) were diluted at a 1μl: 700μl in 5% BSA in TBST. Anti-CGI-58 primary antibody (Novus Biologicals NB110-41576, Oakville, ON, Canada) was diluted at 1μl: 1000μl in 2% powdered milk in TBST. For G0S2 we used two antibodies, Anti-G0S2 at a dilution of 1μl: 2000μl N-Terminus (Santa Cruz, California, USA sc-133424) and a dilution of 1μl: 2000μl Internal (Santa Cruz, California, USA sc-133423) in 5% powdered milk in TBST. All primary antibody incubations were at 4°C overnight. All secondary antibodies were conjugated with

horseradish peroxidase and incubated for 1 hour at either a 1µl: 5000µl (ATGL), 1µl: 10000µl (CGI-58) or 1µl: 20000µl (G0S2) dilution. Exposure was in Immobilon Western Chemiluminescent HRP Substrate (Millipore; Billerica, Massachusetts, USA). Image analysis was performed using band density via ImageJ software produced by the National Institutes of Health (Bethesda, Maryland, USA).

Statistics

All comparisons were made using a one-way ANOVA with Student-Newman-Keuls post-hoc test using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California USA). All statistics were performed on our three skeletal muscles in accordance with our *a priori* hypotheses, with reference made to heart tissue within the text. Significance is reported as $p < 0.05$ ±SEM.

RESULTS

Citrate Synthase. Citrate synthase activity for the heart was roughly 3-fold greater than any of the skeletal muscles. When comparing our SOL, RG and WG samples; our results were consistent with what has been measured previously (11), with RG demonstrating the highest maximal activity (Table 2) ($p=0.01$).

Table 3. Maximal citrate synthase (CS) activity in $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet wt.

	SOL	RG	WG
CS activity	22.3±2.3[a]	34.9±1.9[b]	14.7±1.8[a]

Results are mean±SE (n=8). SOL, soleus; RG, red gastrocnemius; WG, white gastrocnemius. Means designated with the same letter are not significantly different ($p<0.05$).

ATGL, CGI-58 and G0S2 mRNA. Real-time PCR analysis quantified mRNA from SOL, RG, and WG muscles. ATGL, CGI-58 and G0S2 mRNA content was not significantly different across our skeletal muscles (Figure 1). When assessing heart tissue mRNA, ATGL and CGI-58 mRNA was almost 2-fold greater than the WG (data not shown).

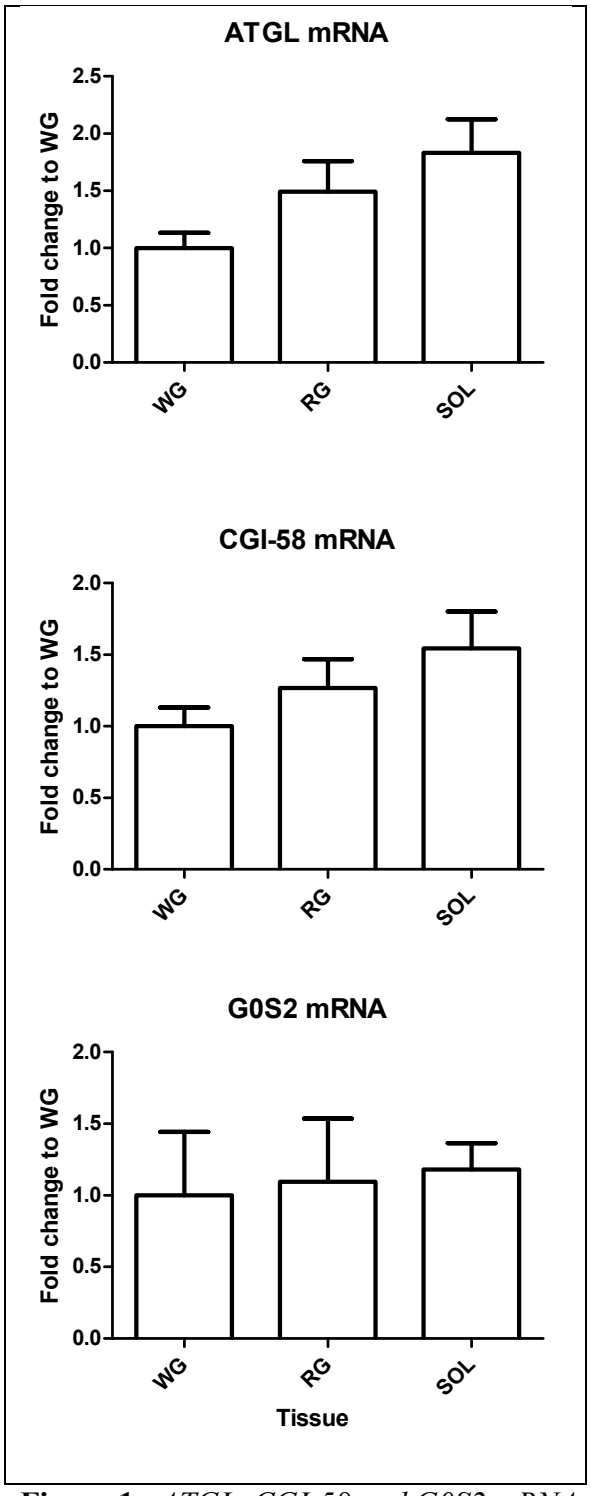

Figure 1. *ATGL, CGI-58 and G0S2 mRNA contents in the three skeletal muscles (SOL, RG, and WG,* n=9*).* Data were calculated according to the delta delta C_T method relative to WG as explained in Materials and Methods. Data are reported as mean±SE , and there were no statistically significant differences ($p<0.05$). SOL, soleus; RG, red gastrocnemius; WG, white gastrocnemius.

ATGL and CGI-58 protein content. SOL had approximately 2-fold greater ATGL protein content compared to WG ($p=0.04$) with RG not being significantly different from either the SOL or the WG (Figure 2, top panel). The range of co-activator CGI-58 protein expression (Figure 2, bottom panel) was markedly different from mRNA expression (Figure 1, middle panel). RG and WG had the highest CGI-58 protein content, with the SOL having the least. Heart and SOL had similar CGI-58 content.

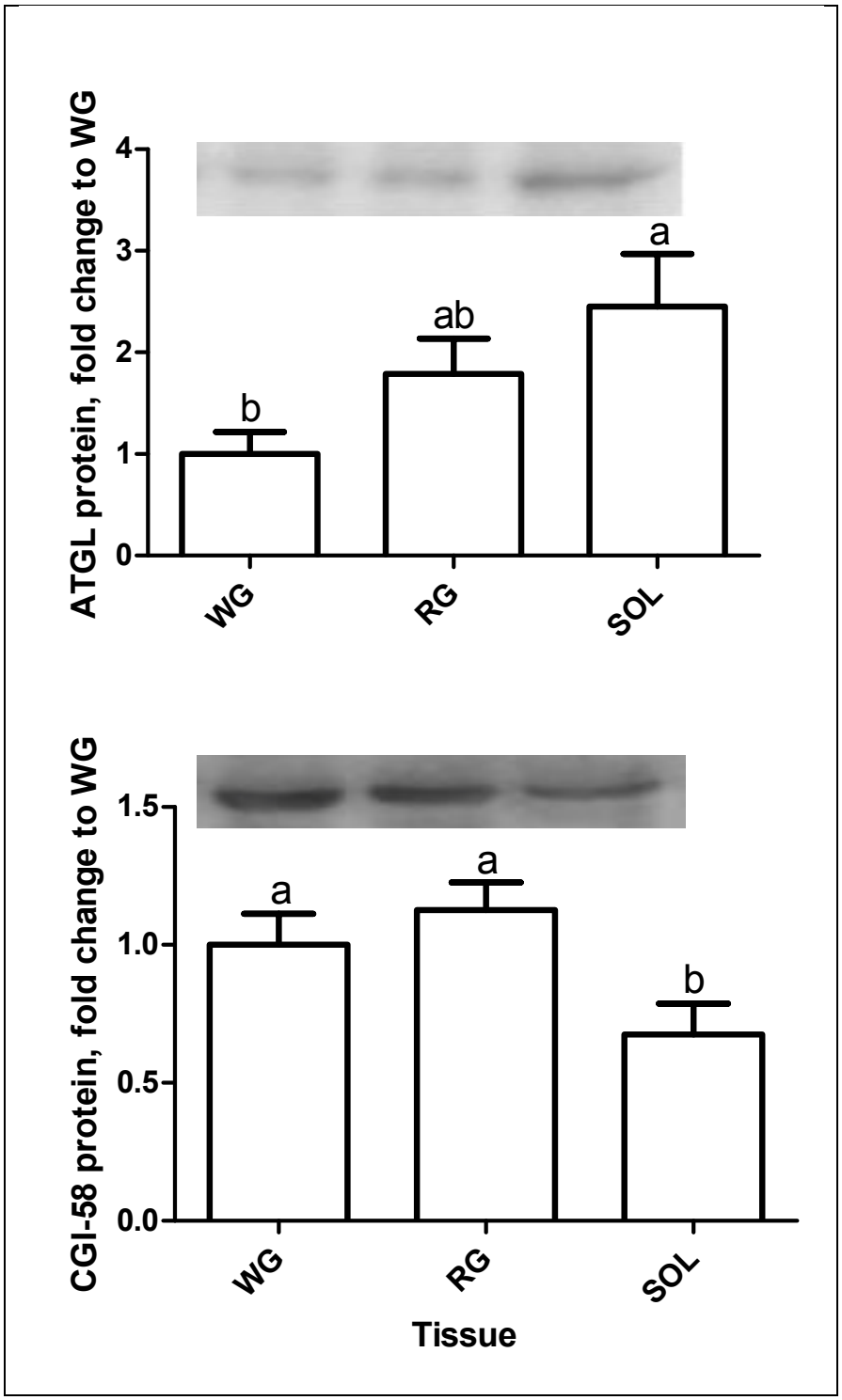

Figure 2. *ATGL* (n=8)*and CGI-58* (n=7) *protein content in the three skeletal muscles (SOL, RG, and WG).* Data are reported as mean±SE and bars with the same letter are not significantly different ($p<0.05$). Insets: representative blots: lane 1, WG (white gastrocnemius); lane 2, RG (red gastrocnemius); lane 3, SOL (soleus).

G0S2 protein content. Although G0S2 has a predicted molecular weight of 11 kDa, we observed two bands that appeared to be G0S2 at 15 kDa and a very strong band at 25 kDa. To determine if both the 15 kDa and 25 kDa bands were G0S2 protein we used two antibodies raised against different regions of the protein (Santa Cruz, California, USA, sc-133423, sc-133424), the N-terminus and an internal region, respectively. Both antibodies detected the 25kDa and the 15kDa protein band, but when specific blocking peptides were pre-incubated with the antibodies (N-13: sc-133424 P, Santa Cruz, California, USA. Internal: sc-133423 P, Santa Cruz, California, USA) both bands disappeared or were significantly reduced (Figure 3). Both the 25kD and the 15kDa band was noticeable in both rat and mouse, with the blocking peptide eliminating both bands in both species, suggesting that the antibodies are specific to both bands.

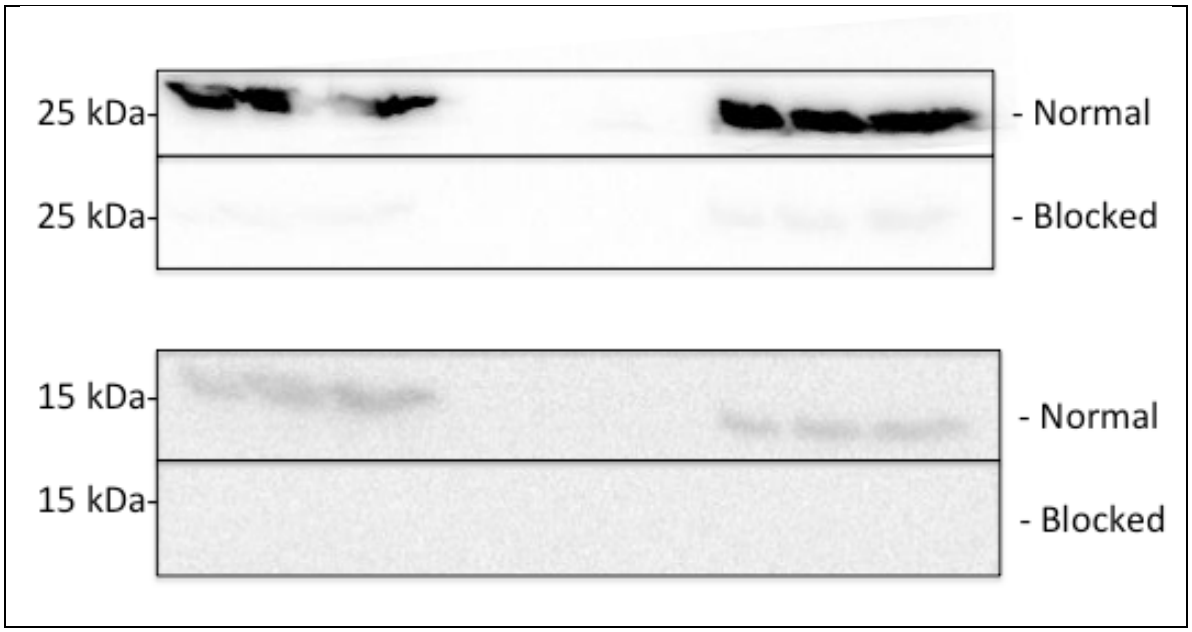

Figure 3. *Establishing that G0S2 protein migrated at both 15 kDa and 25 kDa.* As described in Methods, blots were detected with both antibodies (Santa Cruz, sc-133423, sc-133424) but for the 'Blocked' panel, but specific blotting peptides were used (Santa Cruz, sc-133423 P. sc-133424 P). Images were captured at the same time to eliminate bias placed upon exposure times and image editing. Three lanes on the left were mouse SOL, three lanes on the right were rat SOL. Notice both the

25kDa and the 15kDa band show in both species, and both bands disappear with the antibody pre-incubation with specific blocking peptides.

To confirm which of the bands was G0S2 we acquired mixed quadriceps skeletal muscle of both wild type (WT) and a global G0S2 knockout (KO) mouse (n = 2), a kind gift from Dr. Jun Liu (Department of Pediatrics and the Kentucky Pediatric Research Institute; University of Kentucky; Lexington, KY USA) (24). Following Western blot analyses, the 25kDa band was present in both WT and KO tissues, but the 15kDa was only present in the WT (Figure 4). This confirmed that although the stronger 25 kDa protein was detected by both antibodies, it was not the G0S2 protein. In order to detect the 15kDa band in all tissues, the membrane was cut to eliminate the stronger band at 25 kDa so that the weaker 15 kDa band could be detected.

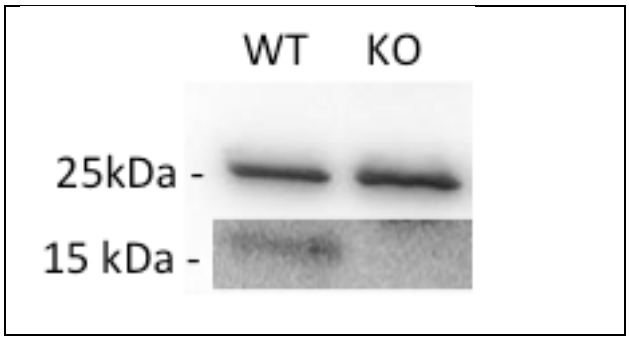

Figure 4. Comparing a wild type to global G0S2 knockout mouse for the G0S2 protein in mixed hind-limb muscle. Notice that in both the WT (wild type) and the KO (knockout) lanes that the 25kDa band is present, yet the 15kDa band is only present in the WT. The top panel represents the 25kDa band, with the bottom panel representing the 15kDa band.

15 kDa G0S2 protein content. The G0S2 protein content in SOL and RG was not significantly different, but both had greater than 2-fold G0S2 protein content compared to the WG ($p=0.001$; Figure 5). The heart was roughly 3-fold greater than the SOL and RG (data not shown).

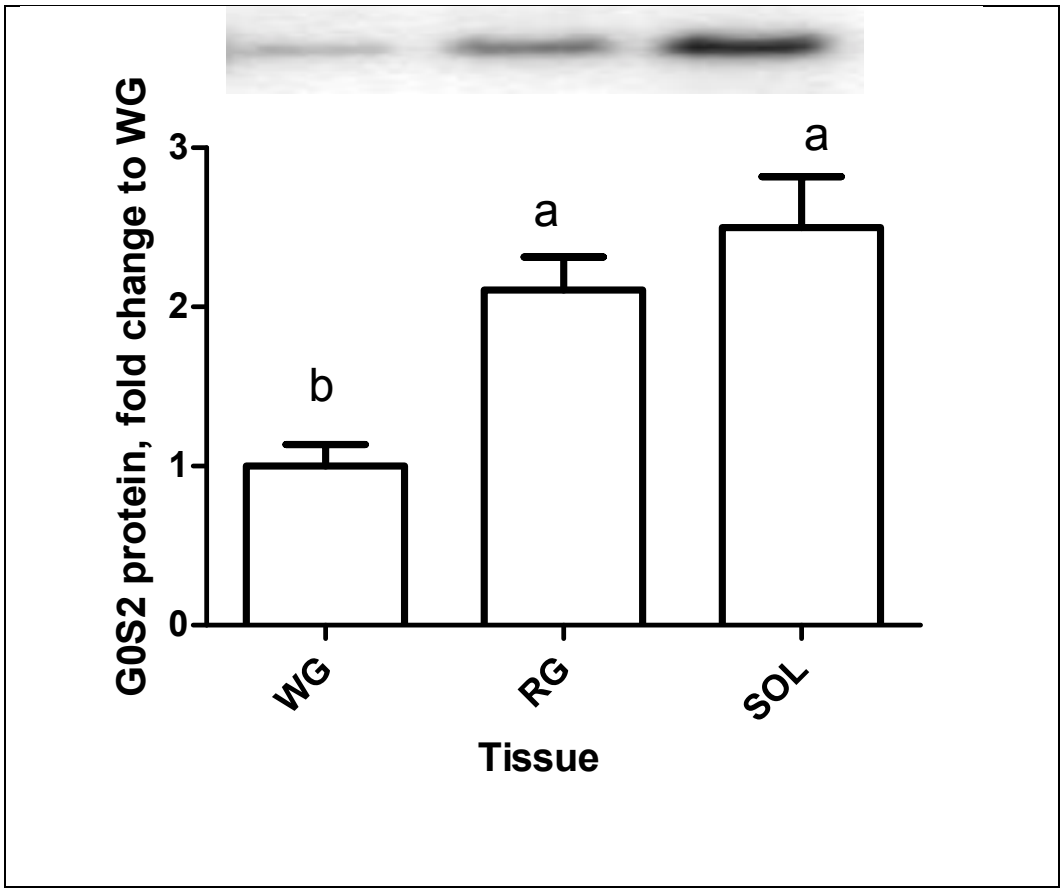

Figure 5. *G0S2 protein content in the three skeletal muscles (SOL, RG, and WG,* n=8*).* Data are reported as mean±SE and bars with the same letter are not significantly different ($p<0.05$). Insets: representative blots: lane 1, WG (white gastrocnemius); lane 2, RG (red gastrocnemius); lane 3, SOL (soleus).

Inhibitor G0S2/ATGL and co-activator CGI-58/ATGL ratios. Due to the importance of G0S2 and CGI-58 in regulating ATGL activity, the ratio of the protein content of the inhibitor and co-activator relative to the lipase content in the three representative muscle fiber types and heart were calculated (Figure 6)**.** Both WG and RG had an approximately 2-fold greater CGI-58-to-ATGL protein ratio when compared to the SOL (Figure 6, top panel), however no significant difference in G0S2-to-ATGL protein ratios were observed between the muscle types **(p=0.11;** Figure 6, bottom panel). Interestingly, heart tissue

had 2-fold greater G0S2-to-ATGL ratio compared to all tissues, yet the lowest CGI-58-to-ATGL (data not shown).

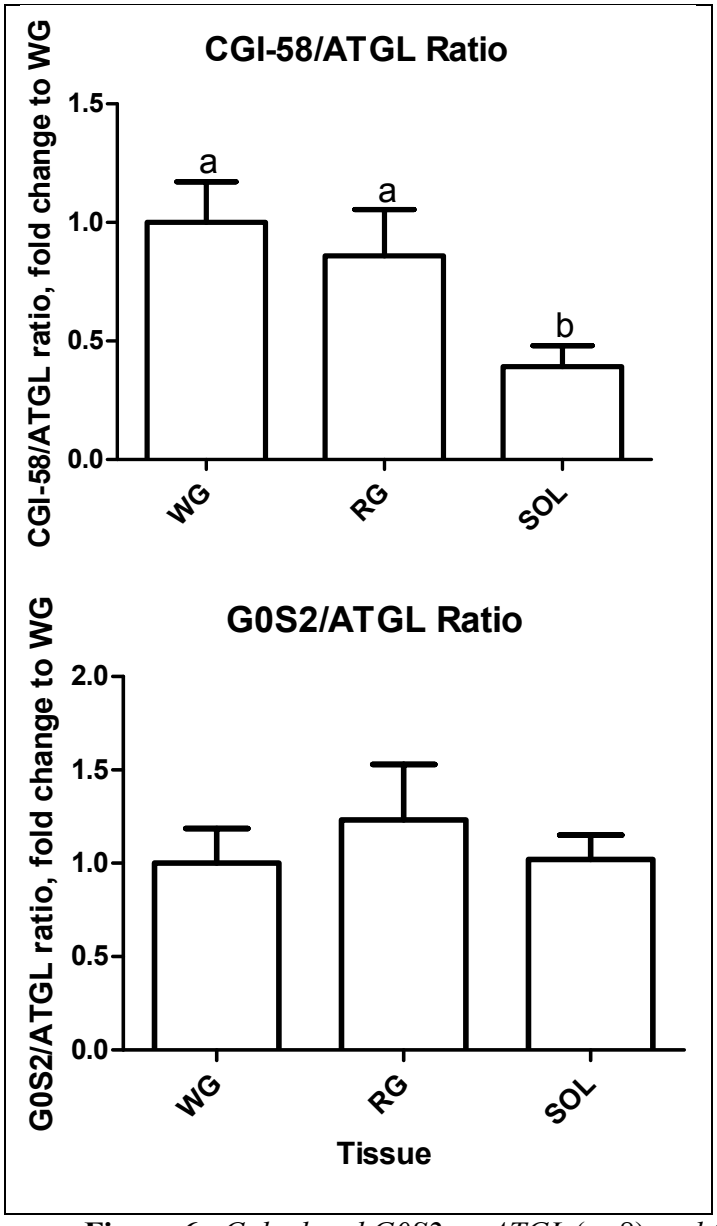

Figure 6. *Calculated G0S2-to-ATGL* (n=8) *and CGI-58-to-ATGL* (n=7) *ratios.* Data are reported as mean±SE and bars with the same letter are not significantly different ($p<0.05$). Please note that ratios were calculated from raw integrations in arbitrary units. Blots were always run in same rat pairs for all the proteins and tissues and every effort to keep exposure times etc., as consistent as possible to keep variation between blots to a minimum. SOL, soleus; RG, red gastrocnemius; WG, white gastrocnemius.

DISCUSSION

This study is the first to compare the mRNA expression and protein content of the primary regulatory lipase (ATGL) with its recently identified inhibitor (G0S2) as well as its co-activator (CGI-58) in non-adipose tissue, such as a model of mixed skeletal muscles. To further characterize the expression patterns of these proteins, three metabolically heterogeneous skeletal muscles were chosen to represent the three basic fiber types: SOL (primarily type I, slow-oxidative fibers, 84% type Ia; 7% type IIa and 9% type IIb/x), RG (representative of type IIa fibers, 51% type Ia; 35% type IIa and 14% type IIb/x) and WG (primarily type IIb/x fibers, 0% type Ia; 0% type IIa and 100% type IIb/x) (3). Several novel findings have been brought forward: 1) we have characterized ATGL, CGI-58 and G0S2 mRNA and protein content across several metabolically different tissues. 2) The ratios of the inhibitor protein (G0S2) and the co-activator protein (CGI-58) to ATGL in the three fiber types appear to be such that highly oxidative type I SOL had the lowest activator-to-lipase ratio with the WG having the highest. Contrary to our hypothesis, inhibitor G0S2 content was not lowest in the oxidative soleus, but was different between skeletal muscles, suggesting that G0S2 protein content alone does not regulate lipase activity.

ATGL and CGI-58 content in skeletal muscle.

ATGL mRNA and protein content followed similar patterns across our array of metabolically different skeletal muscles. There was the greatest amount of ATGL protein content in SOL, followed by the RG with the least in the WG. This is understandable since it has been previously demonstrated the type I fibers have the greatest capacity for

ATGL expression and activity in keeping with their reliance on intramuscular lipolysis (8) , and soleus has the highest type I fiber content (3).

The mRNA expression of lipase co-activator CGI-58 was similar across our array of skeletal muscles. This, however, did not translate to similar protein content. RG had highest CGI-58 protein expression, which corresponds to high oxidative capacity, however the WG has the second highest (although not statistically different from the RG), suggesting that type II muscles have very high CGI-58 regardless of oxidative capacity and reliance on fat as a fuel. In contrast, in 20-week old mice, Badin et al. (1) determined that the heart had the greatest CGI-58 protein compared to the SOL followed by the mixed gastrocnemius muscle. The reason for the disparate findings is unclear, but could be suggestive of the protein content of CGI-58 being variable based upon the age and/or species examined. When expressed relative to ATGL across the three skeletal muscles, we see that the SOL has the lowest activator CGI-58-to-ATGL ratio, with the WG having the highest in spite of the fact that the SOL has the greatest reliance upon intramuscular triglyceride lipolysis as previously demonstrated (20).

ATGL inhibitor G0S2 mRNA expression and protein content.

A novel finding of this study is that G0S2 protein content in sedentary rat skeletal muscle appears to mirror that of ATGL protein content with the SOL having the greatest protein content of both ATGL and G0S2 (although this difference was not statistically different from the RG). This is contrary to our hypothesis, since it was postulated from previous work in primarily adipose tissue and cultured cells that the level of the inhibitor G0S2 is indicative of lipolytic reliance or potential of the tissue. Yet our data does not support this since the SOL (which relies most upon intramuscular triglyceride energy

metabolism) has the greatest G0S2 protein content compared to the WG, which is very glycolytic in nature. Recently, it has been observed in mouse heart tissue following endurance training-induced cardiac hypertrophy, there was no also change in G0S2 protein content (4). However, this physiological adaptation did not change heart lipid content either, suggesting that the heart remained unchanged in its reliance upon lipid content in response to endurance training (4). However, these investigators saw that following aortic banding, inducing a model of pathological hypertrophy, there was an increase in intracellular triglycerides and diglyceride storage within the heart. This was accompanied by an increase of heart G0S2 protein, potentially indicative of decreased rates of lipolysis. Taken together, this study suggests that in heart tissue G0S2 as an important negative controller of lipolysis (4). However, our interest was in skeletal muscle rather than heart muscle, which in general relies more heavily on intramuscular fuels for energy production.

SOL muscle had the highest amount of the inhibitor G0S2 protein followed by RG, which was in turn higher than WG. These results suggest that, contrary to our hypothesis, muscles with a higher type I fiber content have more inhibitor than muscles with a greater type II content, and the oxidative type IIa RG had more potential for lipolytic inhibition than the glycolytic type IIb WG. Further, when expressed relative to ATGL content, type I fibers (SOL) had the same relative amount of G0S2 than both type II muscles (RG and WG). This seemed surprising as SOL relies very heavily on intramuscular triglyceride lipolysis and fat oxidation for energy production (7, 18). Indeed, previous work has illustrated that during exhaustive swimming the SOL was the most reliant on intramuscular triglycerides compared to RG and plantaris, which in turn

were higher than WG (20). Given this, it would appear that the fiber type that is most dependent on lipolysis has the highest level of potential inhibitory control as well. Therefore, it is not clear why SOL, with its high reliance on intramuscular lipolysis, contains the highest protein content of the inhibitory G0S2. However, in pulse-chase experiments, Dyck et al. (6) indicated that the SOL muscle appeared to be very well regulated in terms of switching to lipolysis in the face of lower exogenous FFA during contraction compared to other metabolic fiber types. In addition, in response to endurance training, rat SOL muscle increased intramuscular triglyceride synthesis, and reduced lipolysis, which could argue for a higher level of control that could be mediated by G0S2 (5, 6). Alternatively, if G0S2 has a more important role in regulating mitochondrial function through either positive regulation of oxidative phosphorylation (9) or through pro-apoptotic pathways (22) our data is consistent with this, given that the more oxidative SOL and RG had the highest G0S2 content.

In summary, it would appear that G0S2 is expressed differently in tissues of varying oxidative capacity and reliance on intracellular lipolysis. However, our results do not support the notion that protein content of the inhibitor G0S2 relative to ATGL content can predict lipolytic potential in skeletal muscle. Therefore, it is clear that regulation of lipolysis is not simply mediated by total content of the important proteins, but through more complicated intracellular or post-translational mechanisms.

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Chapter 5

(Formatted for submission to the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology)

Division of labor:

Patrick C. Turnbull: Involved in experimental design, responsible for endurance training of rats, conducting all experiments and biochemical analysis, troubleshooting methodology, writing and editing of manuscript
Amanda Longo: Conducted DEXA scans, assisted with isoflurane anasthesia
Sofhia V. Ramos: Performed mitochondrial isolations and oil-red O staining for muscle lipid, troubleshooting methodology
Brian D. Roy: Involved in experimental designs, writing and editing of manuscript
Wendy E. Ward: Involved in experimental designs, writing and editing of manuscript
Sandra J. Peters: Academic supervisor, involved in experimental designs, troubleshooting methodology, writing and editing of manuscript.

Fiber type specific increases in skeletal muscle adipose triglyceride lipase and its inhibitor G0S2 following endurance training in male Sprague-Dawley Rats.

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Running Head

ATGL and G0S2 following endurance training

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ABSTRACT

Lipolysis is a series of cascading catalyzed reactions resulting in the complete degradation of a triglyceride molecule caused by the release of three fatty acids and a glycerol molecule. Adipose triglyceride lipase (ATGL) is believed to be the first and rate-limiting enzyme in lipolysis. It is co-activated by comparative gene identification-58 (CGI-58) and inhibited by the G(0)/G(1) switch gene-2 protein (G0S2), inhibition is believed to be in a dose-dependent manner. G0S2 has recently been hypothesized to be a positive regulator of oxidative phosphorylation within the mitochondria. Following endurance training there is a change in intramuscular triglyceride (IMTG) dynamics, in which there is increased storage of IMTGs, and a believed increased reliance on IMTG fuel during exercise. Male Sprague-Dawley (n=10, starting age 51-53 days) were treadmill trained for 8 weeks starting at 18m/min for 30 minutes ending with 25m/min for 1 hour, all at a 10% incline. Under isoflurane anesthesia, a sciatic nerve stimulation inducing lipoytic hindlimb muscle contraction was administered for 30 minutes to one leg, leaving the opposing leg as a resting control. The soleus (SOL, mostly type I fiber), red gastrocnemius (RG, mix of type I and type IIa fibers) and white gastrocnemius (WG, no type I fibers, mostly type IIb with some type IIa fibers) were removed. ATGL protein content increased in all three muscles due in the trained condition, with the most pronounced increase witnessed in the glycolytic WG. No change was observed in the co-activator CGI-58 protein content, with increases in inhibiting G0S2 protein only in the trained SOL and trained RG muscles, and with no changes due to acute contraction. In isolated mitochondria from the RG, G0S2 protein content mirrored whole tissue, only increases in the trained group, with no differences following acute stimulation. In

summary, ATGL protein increases in all tissues, with the most pronounced change in the least oxidative tissue, however G0S2 only increased in the oxidative tissues, with no change in the highly glycolytic WG. As well as, no decrease was observed following an acute stimulation suggesting that inhibition of ATGL through G0S2 is not through a dose dependent mechanism, but through more complicated intracellular mechanisms.

INTRODUCTION

Adipose triglyceride lipase (ATGL) is thought to be the first, and rate-limiting step in lipolysis. It is responsible for catalyzing the reaction of removing the first fatty acid from a triglyceride molecule releasing the fatty acid for varying metabolic fates and subsequently producing a diglyceride (42). ATGL activity is modulated by two regulatory proteins (22). It is co-activated by association with comparative gene identification-58 (CGI-58), upregulating ATGL catalytic activity by over 20-fold (19), and putatively inhibited by the G(0)/G(1) switch gene-2 (G0S2) protein. It appears that this inhibition can override the co-activating effects of CGI-58 (22). It is thought that ATGL is directly regulated by influence of both of these proteins since the addition or absence of either CGI-58 or G0S2 can directly alter the rates of ATGLs hydrolytic capability (19, 22, 32). It was originally speculated that ATGL and G0S2 are always bound together (22, 40), yet in fasted human adipose tissue, they witnessed an increase in ATGL protein content with a decrease in G0S2 protein content suggesting that there is not a 1-to-1 stoichiometry between ATGL and G0S2 and further exploration is needed (27). In a completely unrelated role, G0S2 has recently been identified within the mitochondria as a positive regulator of complex V (F_0F_1 ATPase) increasing oxidative phosphorylation (17).

In human vastus lateralis, Froberg et al. (9), measured intramuscular triglyceride (IMTG) content before and after exhaustive cross-country skiing for over 4 hours. They discovered that IMTG content was significantly decreased following a single bout of exercise, identifying IMTGs as an important fuel source during endurance exercise. Following endurance training, it is believed that there is an increase in reliance on IMTG stores for energy provision to the muscle. However there are variable results since there is no standard method for identifying lipolytic rates and different studies yield a variety of different results. Following a 12-week endurance training, Hurley et al. (13) noticed that not only was the respiratory exchange ratio lower following training, but there was also a decrease in plasma glycerol and free fatty acids following an acute bought of exercise, suggesting a greater reliance on intramuscular fat stores for energy provision. They also noticed a sparing of muscle glycogen, and a greater drop in IMTG concentration during acute exercise following training, as well as a greater total stored IMTG following training. Taken together, this would corroborate an increased in muscle IMTG use after endurance training. Increases in reliance on IMTGs can be observed as quickly as 5 days of endurance training, with substantial changes being observed at 31 days (30). The utilization of IMTGs during exercise appear to be fiber type specific, and in Sprague-Dawley rats following exhaustive swimming there was a reduction of IMTG content by 48% in the soleus, which is a predominantly type I fiber tissue, a 29% reduction in the red gastrocnemius, which is a predominantly mixed type I and type IIa fiber, with no reduction in IMTG content in the white gastrocnemius, which is almost entirely type IIb (34). Therefore, it appears that the oxidative SOL and RG have a higher reliance on IMTG during endurance exercise compared to the WG.

It has been demonstrated that ATGL protein content in human mixed fiber vastus lateralis skeletal muscle increases with endurance training, with no increase in CGI-58 (2). Recent work has demonstrated that in obese men who underwent an 8-week endurance training protocol, a significant increase in ATGL protein was elicited, with no increase in CGI-58 or G0S2 protein content (21). However, there have been no studies examining the effects of endurance training on G0S2 protein content in healthy skeletal muscle.

To date, no study has examined the effects of endurance training towards a fiber type specific change in ATGL, CGI-58 and G0S2 protein content. Therefore, the purpose of our study was 2-fold: 1) To determine the effects of endurance training in varying skeletal muscles with varying fiber type composition on ATGL, CGI-58 and G0S2 protein content, and 2) To determine the effects of both endurance training and an acute lipolytic contractile stimulation on whole muscle and isolated mitochondrial G0S2 protein content.

METHODS

Animals

Male Sprague-Dawley rats (n = 10; 51-53 day old starting age) from Charles River Laboratories (Quebec, Canada) were used in this study. All animals were housed in a reverse 12:12 light-dark cycle in the Brock University Animal Facility. All animals had access to *ad libitum* food and water at all times. All experimental protocols and procedures were approved by Brock University Animal care and Utilization Committee and conformed to all Canadian Council on Animal Care guidelines (28).

Training Protocol

Rats were randomly assigned to either the trained condition, or the control condition (n = 10). Trained rats started at 18 m/min for 30 minutes and by week 8 were at 25 m/min for 1 hour, all training was done at a 10% incline (7, 8). Rats were caged in pairs with all cage mates in the same condition (either trained or sedentary). Food intake and body weight measurements were taken once a week. Food intake was measured by combined cage food consumption taking the delta between known starting food weights and food weight 7 days later.

Anesthesia

Rats were anaesthetized by isoflurane inhalant (induced at 5%, monitored between 3-5% during surgical procedures).

DEXA Scanning

Three days before the euthanasia, fat, lean and bone mass were measured using dual energy X-ray absorptiometry (DEXA) (pSabre, Orthometrix). Under isoflurane anaeasthesia, rats were placed supine with all limbs stretched onto the DEXA and a region of 10cm wide by 4 cm long starting from the proximal heads of the femur up towards the thoracic vertebra. All scans were analyzed with a specialized software (Host Software version: 3.9.4; Scanner Software version: 1.2.0) and scanned at a speed of 20 mm/s with a resolution of 0.5 X 0.5 mm.

Sciatic nerve stimulation

During isoflurane-induced anesthesia, sciatic nerve stimulation was applied to stimulate the left leg while the right leg served as a resting internal control (11, 36).

Briefly, a pulse stimuli was applied once every 3 seconds for 15 minutes, followed by a 4 minute rest with no stimulation applied with another 15 minutes of a pulse stimuli every 3 seconds.

Muscle excision

Immediately after 30min sciatic stimulation, the stimulated leg was the first leg for muscle excision followed by the rested leg. The soleus (SOL), the red gastrocnemius (RG) and white gastrocnemius (WG) were all removed and immediately snap frozen and kept in liquid nitrogen, with the plantaris removed and mounted in cryomatrix and placed in liquid nitrogen until stored at -80^{o}C until analysis.

Mitochondrial isolation

There was sufficient RG muscle tissue to extract mitochondria in our samples, and because there were increases in whole muscle G0S2 in response to training in RG, this was used to explore whether there were mitochondrial changes as well. Subsarcolemmal mitochondrial isolation has been previously described and characterized for purity in our lab (31) and adapted from previous studies (14, 29, 37). Briefly, fresh RG tissue was minced manually on ice. Following mincing, samples were placed in 20X (v/w) of first solution (100mM KCl, 40mM Tris HCl, 10mM Tris base, 5mM $MgSO_4$, 5mM EDTA and 1mM ATP) and further homogenized in glass potters. When homogenized, samples were centrifuged at 700g for 10 minutes, with the supernatant being collected and spun again at 14,000g to pellet suspended mitochondria. The subsarcolemmal mitochondrial pellet was resuspended in our 2nd solution (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4, .01mM EDTA, 1% BSA and 0.25mM ATP) and our 3rd solution (100mM KCl, 40mM Tris HCl, 5mM Tris base, 1mM MgSO4,

.01mM EDTA, and 0.25mM ATP) and spun at 7000g for 10 minutes. Our final purification step was conducted using a 60% Percoll ® (P1644, Saint Louis, MO) gradient with final re-suspension in a solution comprising of sucrose and mannitol (220mM sucrose, 70mM mannitol, 10mM Tris HCl and 0.1mM EDTA). Samples were stored in a -80°C freezer until analysis.

Oil red-O neutral lipid staining

Plantaris muscles, embedded in cryomatrix, were cut in 10μm slices and mounted onto glass microscope slides. This protocol is adapted from Koopman et al. 2001 (18), and has been conducted in our lab previously (23-25). Briefly, samples were fixed in 3.7% formaldehyde solution for 1 hour followed by a 30-minute incubation in a working solution of oil red-O (ORO; O0625; Sigma-Aldrich, St. Louis, MO). After washing, slides were coated with 10μl of Prolong Gold Anti-fade reagent (Invitrogen, Carlsbad, CA, USA). Images were captured on a Nikon Eclipse 80i fluorescence microscope (Nikon Eclipse 80i; Chiyoda-ku, Tokyo, Japan) attached to a Retiga 1300 microscope (QImaging, Burnaby, BC, Canada) at 550ηm fluorescence. Images were viewed under 40X magnification, with three muscle sections per tissue and outlined to determine fluorescence using NIS-Elements AR3.00 imaging software (Nikon Instruments, Melville, NY). Data was expressed as a fraction of total stained measured area.

Citrate Synthase

The SOL, RG and WG from the rested (non-stimulated leg) were assayed for citrate synthase activity, as previously conducted in our lab (20). Briefly, tissue was homogenized in 1 M K_2HPO_4 buffer (pH = 8.1) and underwent two freeze/thaw cycles. In a cuvette; triton, acetyl CoA, oxaloacetate and the tissue homogenate were added, free

CoASH produced reacted with 5,5′-dithiobis-2-nitrobenzoic acid. All analyses was conducted in at 412 nm in a GE Ultrospec 2100 pro spectrophotometer (Baie d'Urfe, Quebec, Canada) (35).

Western Blotting

All Western blotting procedures have been previously optimized and described in our lab (Chapter 3). Briefly, SDS-PAGE was performed using 8% (for CGI-58), 10% (for ATGL) or 15% (for G0S2) running gels. Electrophoresis was performed for 85 minutes at 120V and transferred onto 0.45μm polyvinylidene difluoride membrane for ATGL and CGI-58 (Amersham Biosciences, Piscataway, NJ) or 0.20μm polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) for G0S2. Anti-ATGL primary antibody (Cell Signaling Technology, Beverly, Massachusetts, USA cat#2439s) were diluted at a 1μl: 700μl in 5% BSA in TBST. Anti-CGI-58 primary antibody (Novus Biologicals, Oakville, ON, Canada, cat#NB110-41576) was diluted at 1μl: 1000μl in 2% powdered milk in TBST. Two antibodies were used for G0S2 incubation, Anti-G0S2 at a dilution of 1μl: 2000μl N-Terminus (Santa Cruz, California, USA cat#sc-133424) and a dilution of 1μl: 2000μl Internal (Santa Cruz, California, USA cat#sc-133423) in 5% powdered milk in TBST. Band density was determined using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). All blots were made relative to Ponceau stain (Sigma, St. Louis, MO, USA) staining as a loading control.

Statistics

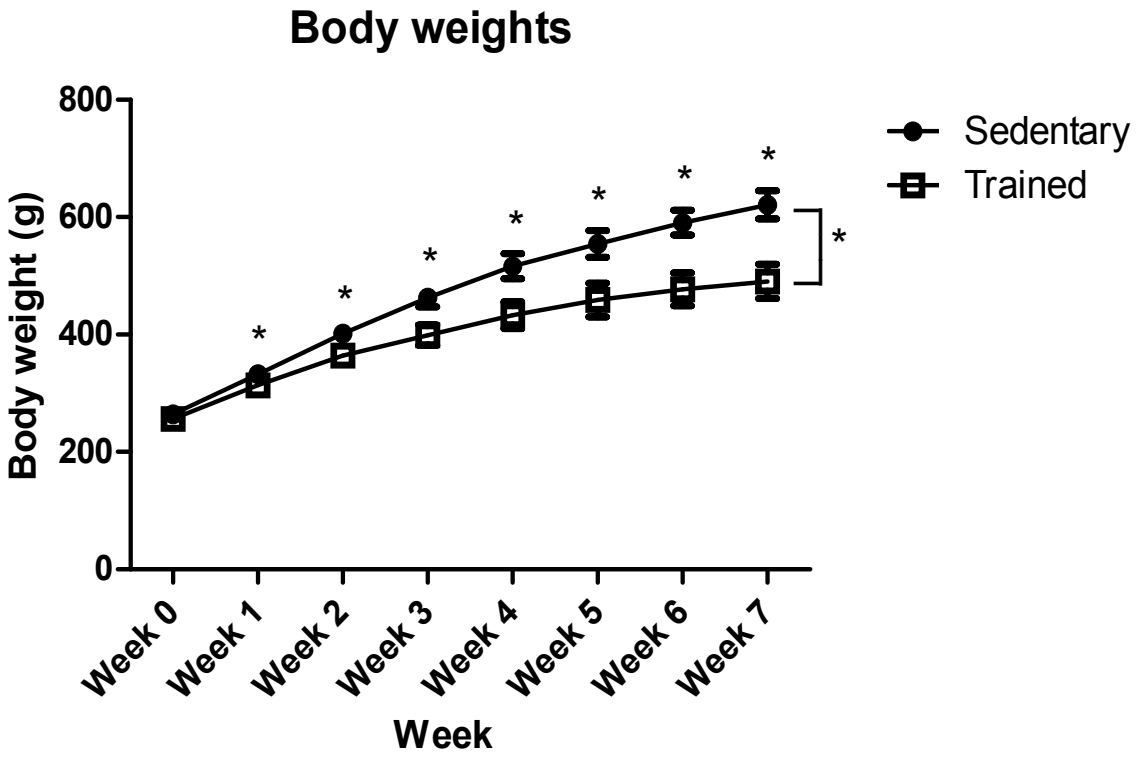
Body weights, composition, and food weights were analyzed as a two-way analysis of variance (2-way ANOVA) for training status x time (week). SOL and RG G0S2 protein content (training status (trained vs. sedentary) x contraction status (rested

leg vs. stimulated leg) and were analyzed using a two-way analysis of variance (2-way ANOVA) with a Student-Newman-Keuls post-hoc test on Sigma Stat (Systat Software Inc., San Jose California, USA). All other statistics were conducted within a given muscle (e.g., SOL, RG, WG) using a student's t-test (trained vs. sedentary) and graphs were prepared on GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California USA). All graphical representation of Western blot data is made relative to the average of the rested sedentary control so that significance does not change when compared to arbitrary units. Significance is reported as $p < 0.05$.

RESULTS

Body weights

Although both sedentary and trained groups increased in size, the sedentary group was consistently heavier than the trained group from week 1 to week 7. ($p<0.001$). (Figure 1)



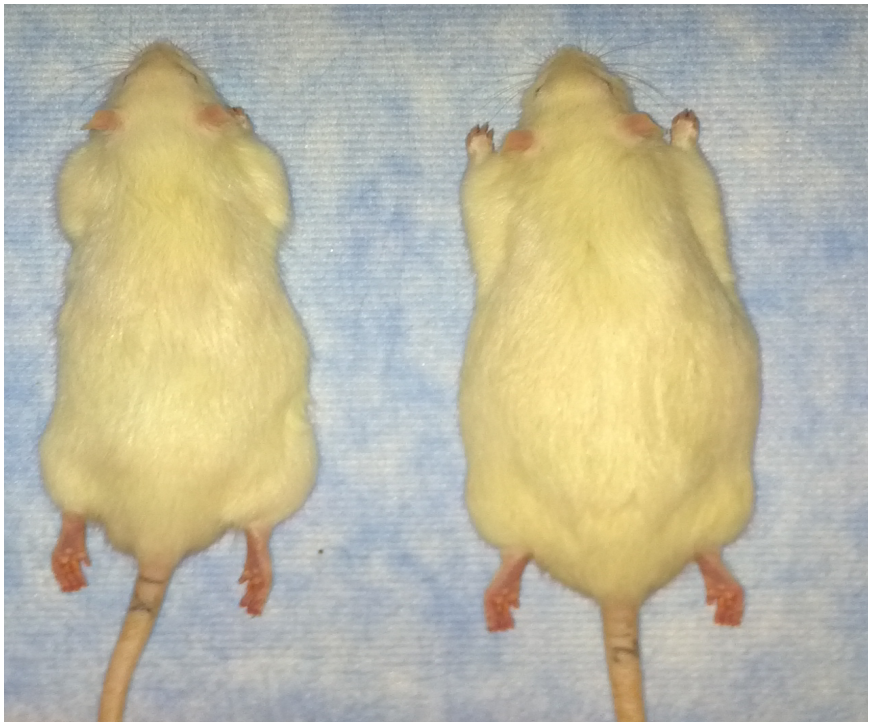


Figure 1a. Body weights of sedentary compared to trained rats from week 0 to final body weights one week before final surgeries (n=9). Weights were recorded once a week on the same day for all animals on a pan balance. Data are reported as mean±SE, *, signifies significant differences between trained and sedentary for each individual

week. There were significant differences for every week compared to each other both in sedentary and trained ($p<0.05$). Sedentary are expressed as the closed circles, trained are expressed as the open squares. B. Photo of a representative rat from both the trained (left) and sedentary (right) group closest to the mean body weights at week 5.

Food Weights

The trained group ate significantly less than the sedentary group throughout the duration of study starting at the first week ($p<0.001$; Figure 2).

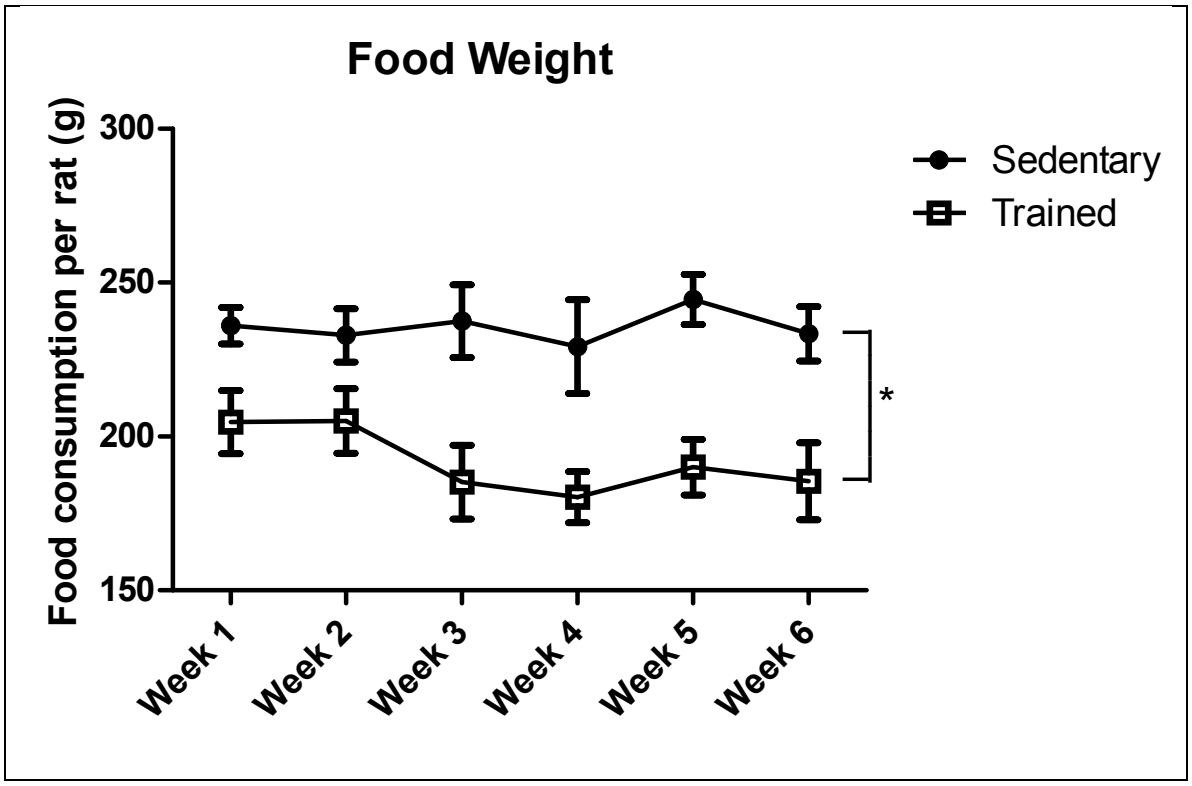


Figure 2 Food weights of sedentary compared to trained rats from the end of week 1 until the end of week 6. Food weights are a measure of the difference from starting food weight compared to end food weight per cage, then divided by two (n=9). Data are reported as mean±SE. *, signifies significant differences between trained and sedentary for each individual week ($p<0.05$). Sedentary are expressed as the closed circles, trained are expressed as the open squares.

Body composition (DEXA)

Lean mass, fat mass and bone mineral content (BMC) were added together to create a relative region of interest (ROI). Lean mass, fat mass and BMC were made relative to ROI as a percentage of total area measured. There was a 1.3-fold increase in lean mass in the trained group compared to sedentary group ($p<0.001$, Figure 3 – top panel). There was a greater than 4-fold decrease in fat mass in the trained group compared to the sedentary group ($p<0.001$, Figure 3 – middle panel). BMC was not significantly different between groups (Figure 3 – bottom panel). Bone mineral density (g/cm^2) was roughly 10% greater in the trained group compared to sedentary ($p=0.03$, Figure 4).

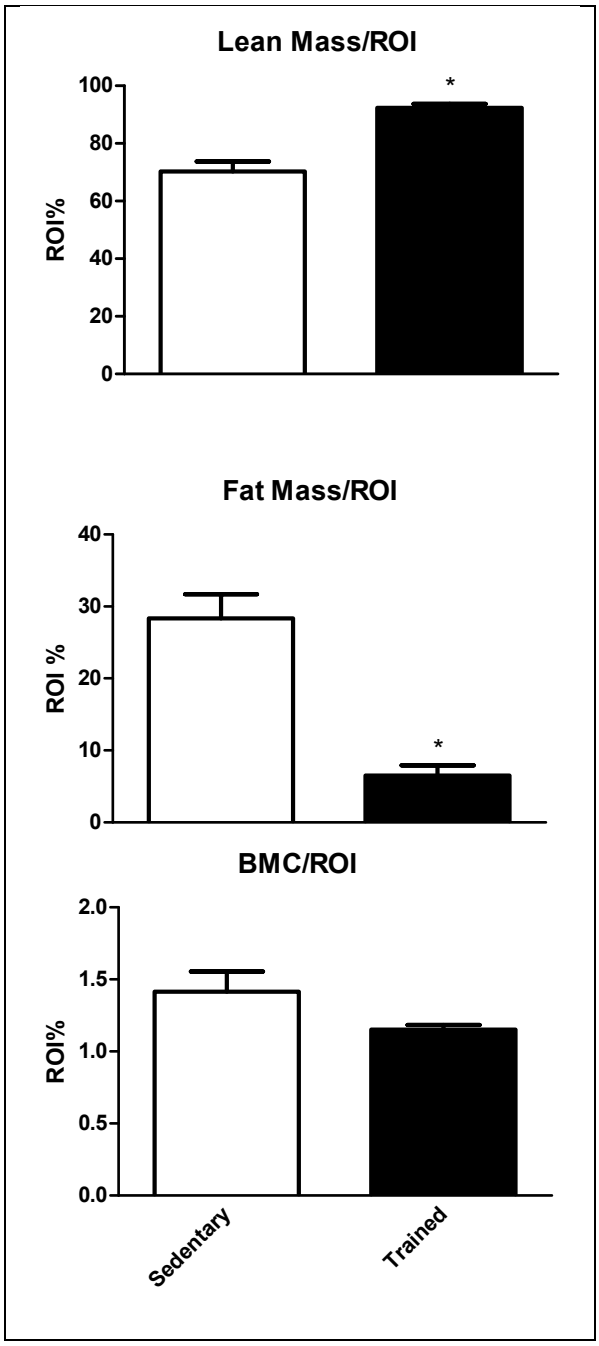


Figure 3 Lean mass, fat mass and bone mineral content (BMC) were measured via DEXA (n=9). Scans were done by located proximal heads of the femur and expanding caudally until scan window was full, analysis was conducted on our collected region of interest (ROI) which comprised of our lean mass + fat mass + BMC. Data are reported as mean±SE. *, signifies significant differences between trained and sedentary ($p<0.05$). The sedentary rested group is expressed as the open bars, with the trained rest group the closed black bars.

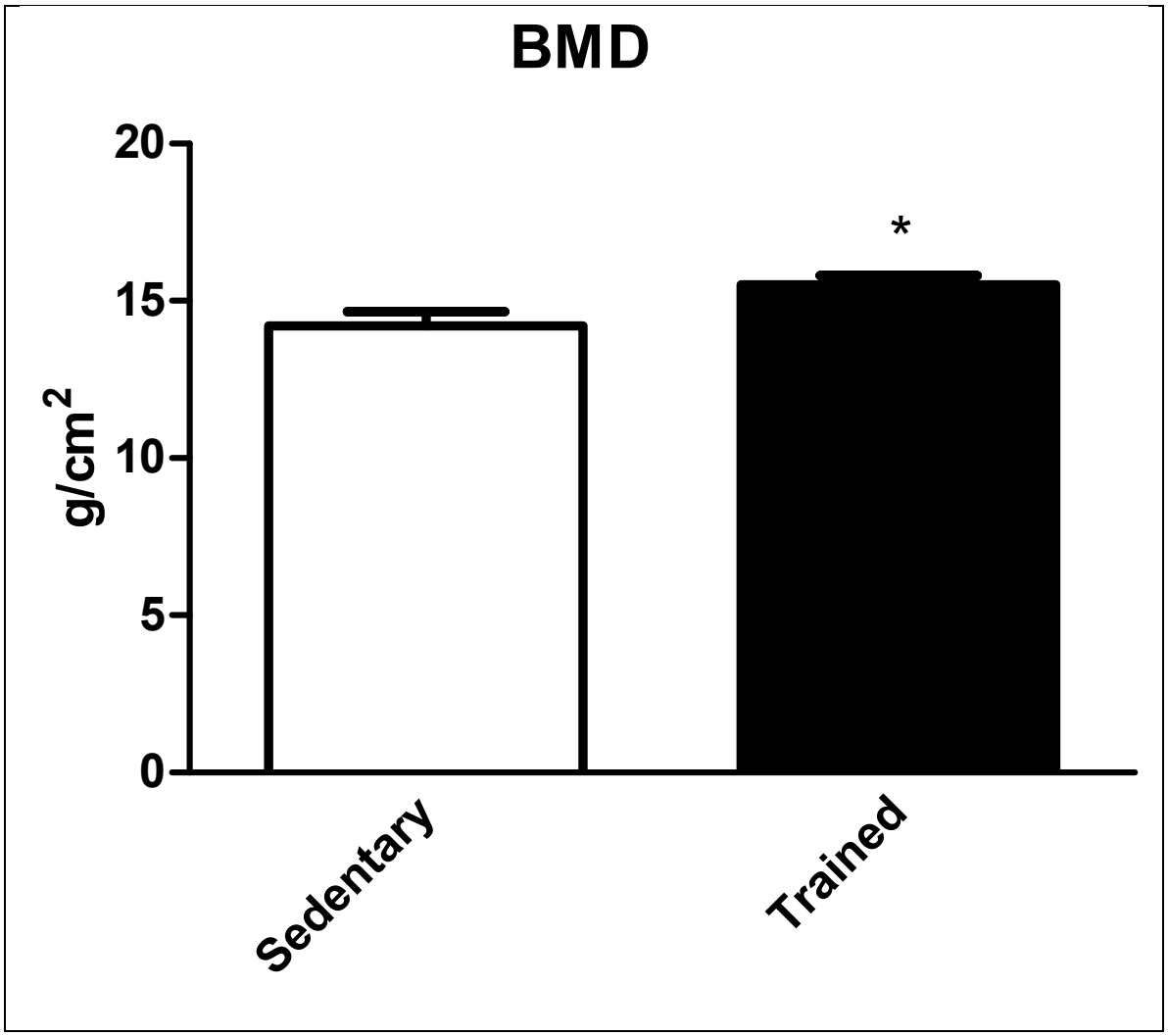


Figure 4 Bone mineral density (BMD) was collected using DEXA (n=9). BMD is an absolute measure in g/cm$^{2.}$ Data are reported as mean±SE. *, signifies significant differences between trained and sedentary ($p<0.05$). The sedentary rested group is expressed as the open bar, with the trained rest group the closed black bar.

Citrate Synthase

As expected, prior to training, SOL and RG had similar CS activity, which was higher than WG. As a result of endurance training, CS activity increased in all three muscles such that SOL>RG>WG (Table 1).

Table 4. Maximal citrate synthase (CS) activity in $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet wt.

CS Activity	Sol	RG	WG
Sedentary	32.5 ± 1.8^{a}	29.4 ± 4.5^{a}	15.3 ± 3.1^{b}
Trained	54.8 ± 4.1^{c}	42.1 ± 3.9^{d}	27.9 ± 3.1^{f}

Results are mean ±SE (n=10). SOL, soleus, RG, red gastrocnemius, WG, white gastrocnemius (n=9). Means assigned with the same letter are not significantly different ($p<0.05$).

ATGL protein content

There was a 1.3-fold increase of ATGL protein content in the trained SOL muscle compared to the sedentary group (p=0.02, Figure 5 – top panel), a 1.7-fold increase of ATGL protein content in trained RG compared to the sedentary group (p=0.04, Figure 5 – middle panel) and almost a 2-fold increase in ATGL protein content in trained WG compared to the sedentary group (p=0.04, Figure 5 – bottom panel).

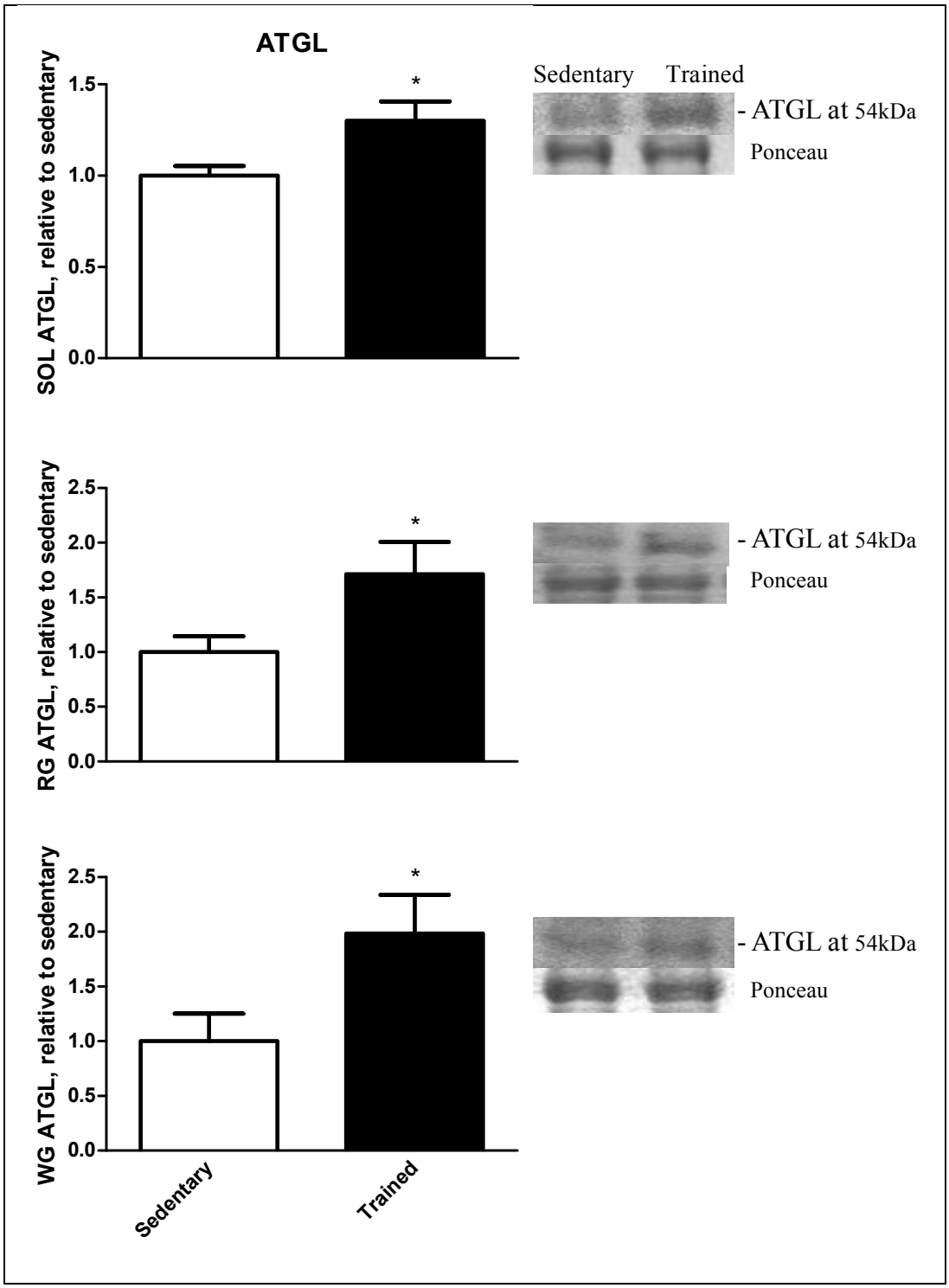


Figure 5. ATGL protein content from our three rested skeletal muscles (SOL, RG, WG, n=9). Data are reported as mean±SE. *, signifies significant differences between trained and sedentary ($p<0.05$). The sedentary rested group is expressed as the open bars, with the trained rest group the closed black bars.

CGI-58 protein content

There were no significant differences in CGI-58 protein content due to endurance training compared to the sedentary group in SOL ($p=0.7$, Figure 6 – top panel), RG ($p=0.7$, Figure 6 – middle panel) or WG ($p=0.8$, Figure 6 – bottom panel).

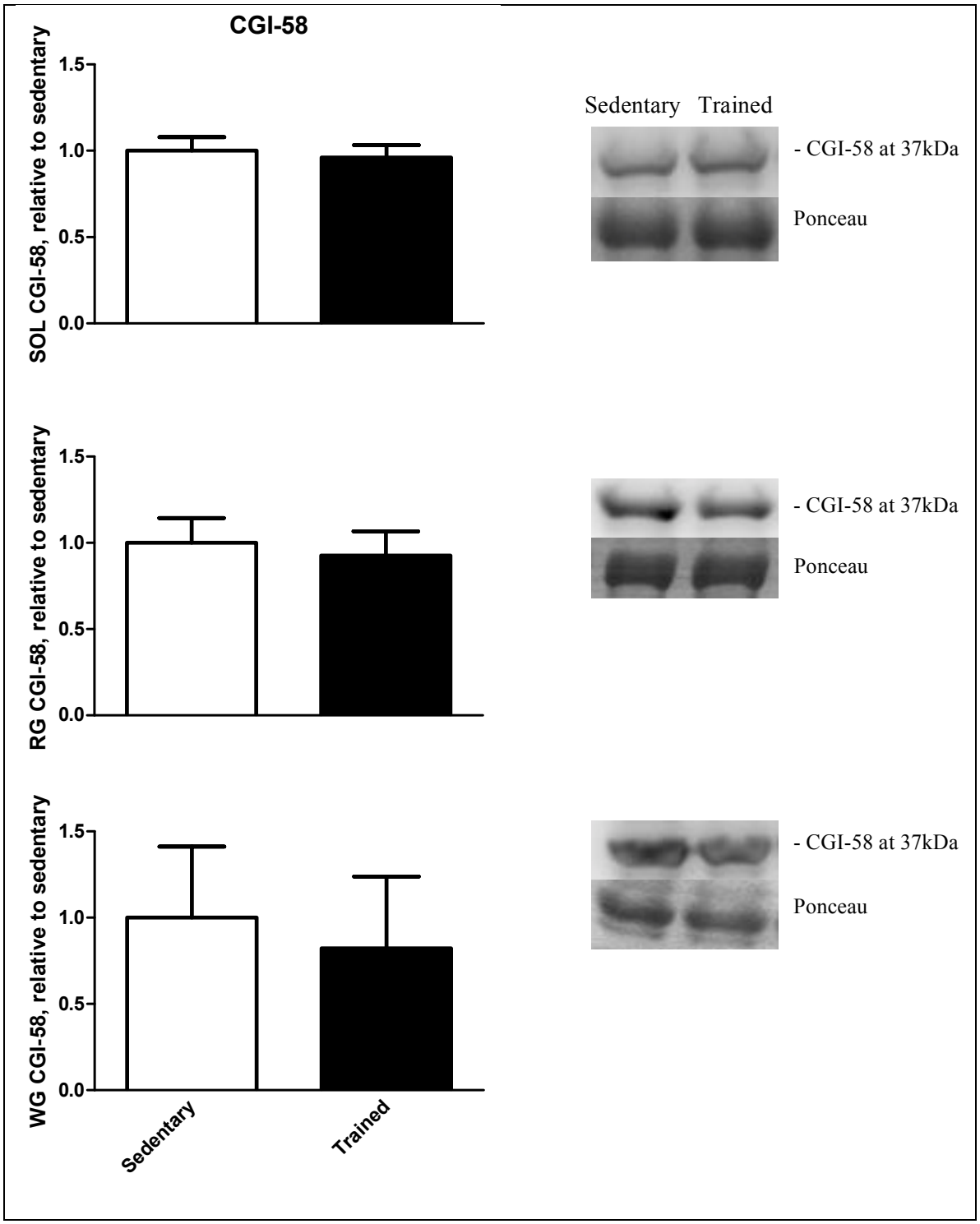


Figure 6 CGI-58 protein content from our three rested skeletal muscles (SOL, RG, WG, n=9). Data are reported as mean±SE. *, signifies significant differences between trained and sedentary ($p<0.05$). The sedentary rested group is expressed as the open bars, with the trained rest group the closed black bars.

Whole muscle G0S2 protein content

There were increases in G0S2 protein content in response to training in the SOL and RG, protein content was assess both for training status (trained vs. sedentary) and sciatic stimulation (rest vs. stimulated leg). A 2-way ANOVA was performed on both SOL and RG with no interaction observed. A main effect was observed for training status with the trained group having a greater G0S2 protein content compared to the sedentary group in both the SOL $p<0.001$, Figure 7 – upper panel) and RG ($p=0.005$, Figure 7 – middle panel) with no main effect discovered for sciatic stimulation. There was no difference in G0S2 protein content in the WG in either the trained group or sedentary group ($p=0.9$, Figure 7 – bottom panel).

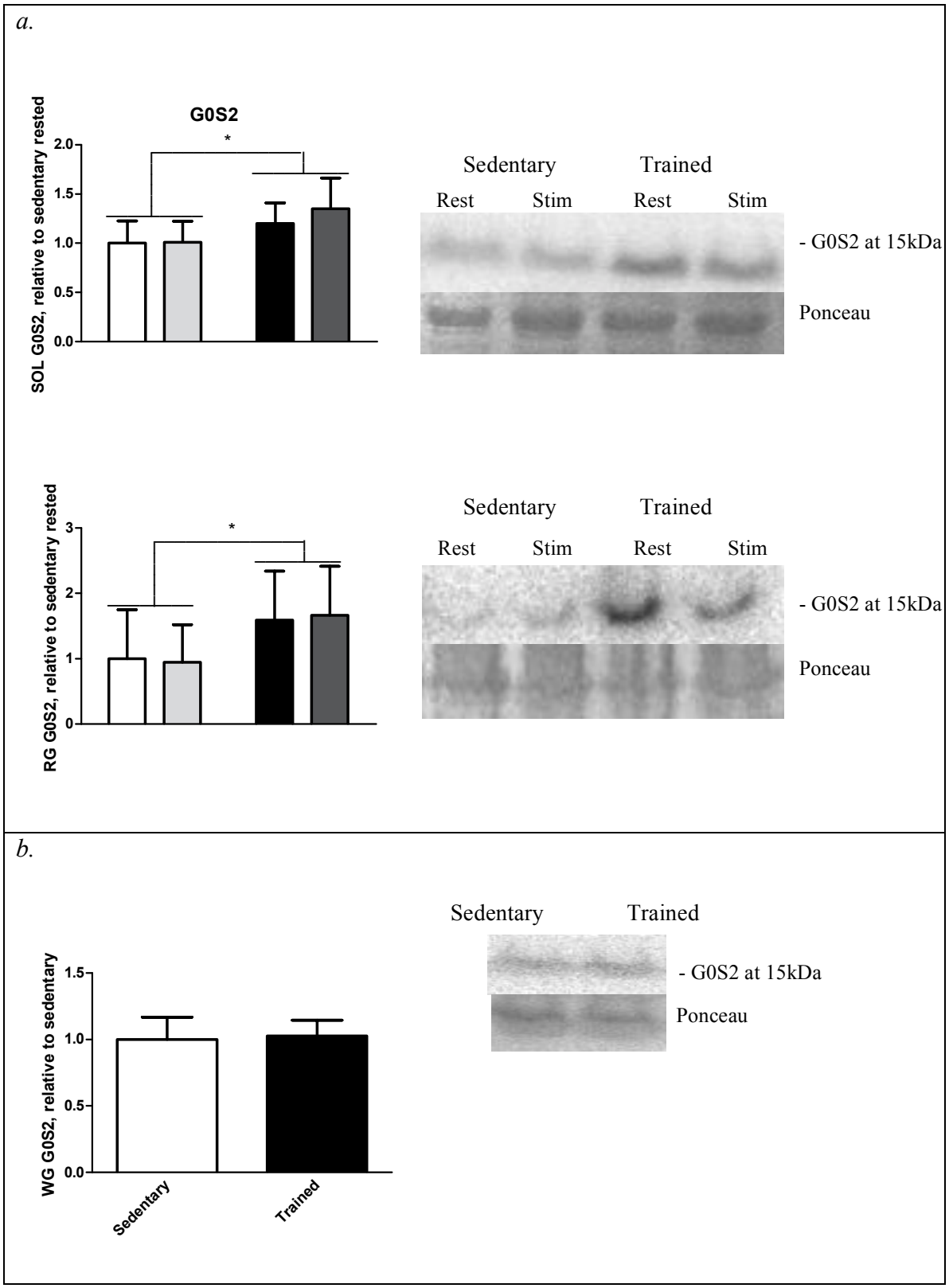


Figure 7 a. G0S2 protein content from both rested and stimulated legs of SOL and RG in both trained and sedentary groups as well as G0S2 protein content of rested WG (n=9). Data are reported as mean±SE. '*' signifies significant differences ($p<0.05$).

The sedentary rested groups are expressed as the open bars, the sedentary stimulated groups are the light grey bars, with the trained rest group the closed black bars and the trained stimulated group are the dark grey bars. **b.** G0S2 protein content in the WG between sedentary and trained. Data are reported as mean±SE.

Mitochondrial G0S2 protein content

Because there were increases in whole muscle G0S2 in RG and there was sufficient tissue to extract mitochondria, we examined whether there was any change in mitochondrial enrichment of G0S2 following training, and whether this was altered by an acute bout of muscle contraction. There was a main effect for training status such that there was a 2-fold increase in G0S2 relative protein content in mitochondria from trained RG muscle ($p=0.01$, Figure 8) compared to mitochondria from sedentary RG muscle, but there were no differences observed with contraction in either group ($p=0.6$).

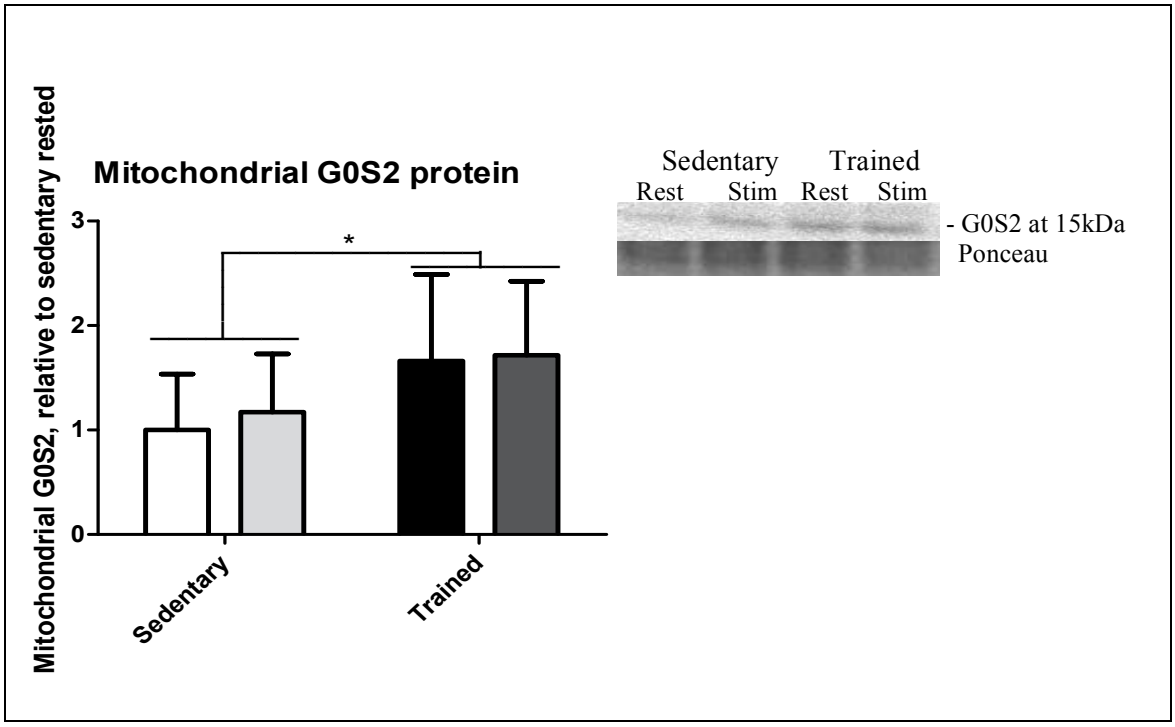


Figure 8. Mitochondrial G0S2 protein content from both rested and stimulated legs of isolated RG mitochondria in both trained and sedentary groups (n=9). Data are reported as mean±SE. *, signifies significant differences between trained and sedentary ($p<0.05$). The sedentary rested group is expressed as the open bar, the

sedentary stimulated group is the light grey bar, with the trained rest group the closed black bar and the trained stimulated group is the dark grey bar.

Muscle lipid content

No interaction was observed, however main effects for both training status and sciatic stimulation was observed. Following stimulation there was a decrease in intramuscular lipid content ($p<0.001$) regardless of training status. However, trained rats had a higher level of intramuscular lipid content compared to sedentary rats, regardless of sciatic stimulation ($p=0.004$, Figure 9).

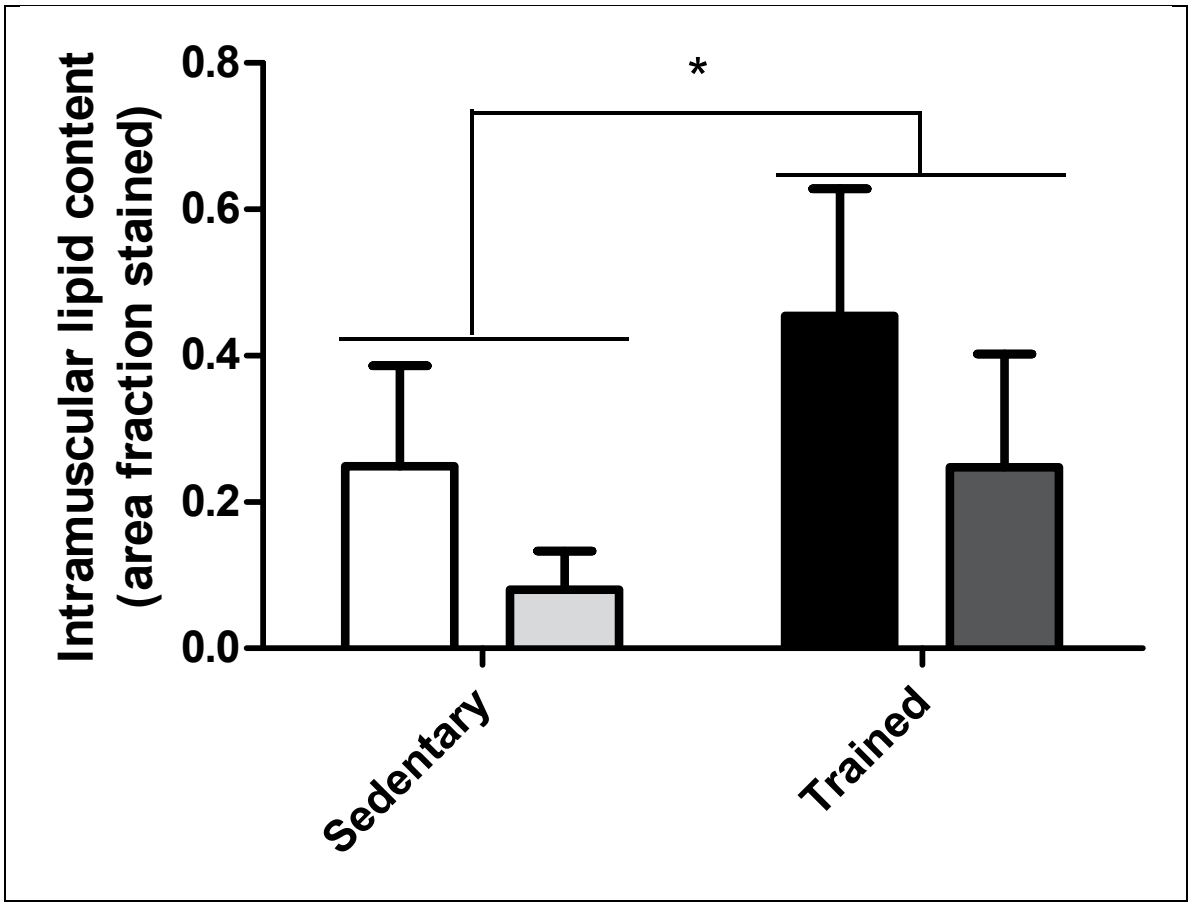


Figure 9. Muscle lipid content from trained and sedentary groups both with and without sciatic stimulation from the plantaris muscle (n=9). Data are reported as mean±SE with an '*' signifying significant differences between trained and sedentary

($p<0.05$). The sedentary rested group is expressed as the open bar, the sedentary stimulated group is the light grey bar, with the trained rest group the closed black bar and the trained stimulated group is the dark grey bar.

Discussion

This study is the first to examine the effects of endurance training in several metabolically different skeletal muscles (soleus, red and white gastrocnemius) on the relative protein expression of adipose triglyceride lipase (ATGL), it's co-activator, comparative gene identification – 58 (CGI-58) and it's inhibitor the G(0)/G(1) switch gene-2 protein (G0S2). Using a multi-muscle model allowed us to examine any potential differences in fiber type as the SOL is primarily a type I (slow oxidative) fiber tissue (84% type Ia; 7% type IIa and 9% type IIb/x), the RG being representative of mostly a mix of type I and type IIa fibers (51% type Ia; 35% type IIa and 14% type IIb/x) and the WG being almost entirely type IIb (0% type Ia; 0% type IIa and 100% type IIb/x) (5). Our oil red-O muscle lipid data supports our sciatic stimulation muscle contraction protocol as a model for inducing muscle lipolysis, although this was unable to show any differences between our trained group and sedentary, perhaps due to the insensitivity of oil red-O staining or perhaps to the contraction stimulus. In voluntary contraction, type I fibers are recruited first during lower maximal voluntary contraction, and as the contraction force increases, type IIa followed by type IIb fibers get recruited (12). With forced electrical stimulation, such as a sciatic stimulation, this does not appear to be the case. It is thought that motor unit recruitment no longer follows a sequential fiber type contraction, but a more random or perhaps even reverse pattern occurs such that type IIb fibers are contracted at the same rate, or perhaps recruited earlier than type I (10). This fixed fiber contraction rate appears to lead to differences in metabolic rate and perhaps

substrate reliance, and significantly decreases time to fatigue with stimulated contraction (reviewed by (26)). Therefore, the sciatic stimulated contraction may not yield demonstrated increases in IMTG reliance due to endurance training since stimulated motor unit recruitment is not consistent with voluntary muscle contraction. Our training protocol did however appear to yield conformational changes despite no apparent changes in IMTG reliance since there was significantly greater lean mass in our trained group compared to the sedentary, with fat mass being the greatest in the sedentary group. When comparing body weights, the sedentary group weighed significantly more every week compared to the trained group after the first week. This is interesting since it appears that the sedentary group ate significantly more compared to the trained group, despite the expected greater energy expenditure in the trained group, therefore a greater need for food consumption. One possible explanation for this is that it has been observed that the predominate feeding time for rats is during the light cycle (4), while our rats were trained during the dark cycle, it can be speculated that perhaps the trained rats required more sleep and prioritized this before eating. Following an acute bout of exercise, it was noticed in human adult males that there was a brief appetite suppression delaying the onset of feeding post exercise, with chronic exercise leading to a negative energy balance such that this appetite suppression led to a significant decrease in energy consumption compared to energy expenditure (16).

Several novel findings have been identified: 1) ATGL protein increase in all three skeletal muscles, yet the largest increase is in WG that historically relies the least on fat as a fuel source; 2) CGI-58 protein content does not change regardless of skeletal muscle and training status; 3) G0S2 protein increases following endurance training, but only in

the SOL and RG, with no change observed in the WG; and 4) RG subsarcolemmal mitochondrial G0S2 protein is increased in the trained group compared to the sedentary, however, there were no observable effects stemming from the lipolytic inducing sciatic stimulation.

ATGL protein content

ATGL protein content was increased in the trained group compared to the sedentary group. This has been reported before (2, 3, 21, 41); however, ATGL protein increased across a variety of skeletal muscle tissues, with the largest increase in the glycolytic WG. Jocken et al. (15) immunostained in human vastus lateralis for ATGL protein and determined that ATGL is only expressed in type I fibers. We have previously demonstrated that ATGL has the greatest expression in SOL, which is predominantly type I fibers, but to a lesser degree ATGL is also expressed in the WG, which has no type I fibers (Chapter 4). The differences in results however, can be accounted to methodological differences since immunohistochemical analysis relies much more on threshold detections compared to western blotting. Therefore, it is understandable that there are increases in ATGL protein content due to endurance training, since there is a fiber type shift in oxidative fiber types with a decrease in type IIb (as reviewed by (33)).

CGI-58 protein content

CGI-58 protein content was unchanged regardless of training status; this was consistent across all of our skeletal muscles. It has been previously demonstrated in several studies that CGI-58 does not change, regardless of training status. Most studies to date have focused on human vastus lateralis, and thus we have added to this information by demonstrating that, regardless of the skeletal muscle preference towards oxidative or

glycolytic metabolism, CGI-58 protein content is unaltered. This is interesting, since previously we have demonstrated that relative CGI-58 protein content is different across the SOL, RG and WG, with the SOL having the least relative amount of CGI-58 compared to the RG and WG (Chapter 4**)**. Perhaps the RG and WG might need to be more sensitive to changes in energy provision, so that the tissue can increase lipolytic rates in situations with increased prolonged energy demands to the muscle.

G0S2 protein content

We are the first to demonstrate that G0S2 protein content increases following training in skeletal muscle, but the effects of training appear to be fiber type specific, since G0S2 protein content only increased in the SOL and RG, and remain unchanged regardless of training status in the WG. Previous work in heart tissue demonstrated that following physiological heart hypertrophy caused by endurance training, there was no increase in heart G0S2 protein or in cardiac lipid storage (6). This was in sharp contrast to pathological heart hypertrophy caused by aortic banding, where there was an increase in triglycerides and diglycerides, as well as an increase in G0S2 protein content (6). This is interesting since porcine adipose tissue G0S2 mRNA expression was significantly greater during adipogenesis (1), which could explain why not only is there an increase in G0S2 protein content due to pathological hypertrophy which increased cardiac intracellular lipid content, but also in our current study, which we determined that we had a greater intramuscular triglyceride content in resting muscle following endurance training.

Interestingly, it has been postulated that ATGL and G0S2 are always bound together (22, 40), therefore an increase in one protein should result in an increase in the

other. However, we have demonstrated that if this is true, then it is not likely a one-to-one stoichiometric relationship and perhaps that there are pools of either ATGL or G0S2 that are separate from each other. Nielsen et al. (27) demonstrated in human adipose tissue following fasting that ATGL protein content increased, while G0S2 protein content decreased, most likely to facilitate lipolytic rates, since there were greater rates of circulatory free fatty acids. This is of particular interest, since not only do they demonstrate that ATGL and G0S2 protein content can have inverse expression, but also it is postulated that in a heightened state of lipolytic demand, ATGL protein content increased to facilitate triglyceride breakdown, and that G0S2 protein decreased to reduce its inhibitory control over ATGL. In a G0S2 overexpressing HeLa cells, Lu et al. (22) discovered that despite CGI-58 supplementation in an attempt to stimulate lipolysis, ATGL was unable to catalyze the conversion of a triglyceride to a diglyceride indicating that levels of G0S2 could regulate ATGL activity. Further support for this came from research using a G0S2 knockdown HeLa cell line, where both basal and stimulated lipolysis was significantly increased (40). Not only does this suggest non-competing mechanisms for co-activation/inhibition, but also that G0S2 protein dose dependently can inhibit ATGL function. This is contradictory to our findings since we had an increase in both ATGL and G0S2 following training in our oxidative tissues, but also that we had no change in G0S2 protein content following a 30 min lipolytic muscle contraction, suggesting that G0S2 protein content alone does not indicate ATGL function in skeletal muscle.

Louche et al. (21) studied the effects of endurance training in obese males on skeletal muscle ATGL and G0S2 protein content. Following training, they observed a

robust increase in ATGL protein content, yet no change in G0S2 protein content. These results are similar to our WG data, in that in our trained group there was increase in ATGL protein content, yet no change in G0S2 protein content. In obese subjects, there is significantly less type I fibers with a greater proportion of type IIb fibers (38), which is a similar fiber distribution to that of the WG, which is suggestive of a fiber type preference for G0S2 protein upregulation due to training.

Mitochondrial G0S2 content

A novel finding from this study is that in isolated RG subsarcolemmal mitochondria, there was an increase in G0S2 protein content in our trained group compared to our sedentary group, however there was no change due to an acute 30 min lipolytic contraction, in either the trained or sedentary group. Two separate studies have identified G0S2 as a specific mitochondrial protein. Welch et al. (39) determined that G0S2 interacts with Bcl-2 promoting apopotosis, and Kioka et al. (17) determined that G0S2 interacts with complex V (F_0F_1 ATPase) positively up regulating ATP production. While we can not comment on the apoptotic effects of G0S2, following muscle contraction that promoted lipolysis, there were no acute changes in mitochondrial G0S2 protein content, therefore suggesting that regulation of complex V by G0S2 is through more complicated post-translational mechanisms, and not necessarily through mitochondrial enrichment of G0S2.

In summary, ATGL protein content increases due to endurance training, and the relative increase in ATGL is greatest in the WG, which typically relies on fat the least for energy production. This however, does not support to co-ordinate expression of G0S2 protein content, since the WG is the only one the three skeletal muscles studied that did

not demonstrate an increase in G0S2 protein content. Within the SOL and RG and isolated mitochondria from the RG, there were increases in G0S2 protein content due to training; however, there were no acute changes due to a lipolytic inducing contraction. This suggests perhaps a chronic regulation of G0S2 protein content. Our study suggests that while G0S2 may have inhibitory effects on ATGL, and alternate effects within the mitochondria, these specific functions appear to be under more complicated control other than protein content alone, perhaps through post-translational or intracellular mechanisms.

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Chapter 6

Integrative discussion

Since the discovery of what is now considered the rate-limiting enzyme of lipolysis, adipose triglyceride lipase (ATGL) in 2004 (7, 19, 24), there have been many studies contributing to our understanding of the regulation of ATGL in adipose tissue. However, significantly fewer studies have focused on ATGL regulation in skeletal muscle. In adipose tissue, lipolysis appears to be regulated predominantly by PLIN1, which is a protein that surrounds adipose tissue lipid droplets (3), regulating the release of CGI-58 to co-activate ATGL activity (4). PLIN1 is absent or in very low concentrations in skeletal muscle, therefore the regulation of ATGL appears to be reliant much more on direct protein regulation through co-activation by CGI-58 (11), and inhibition by the G(0)/G(1) switch gene-2 protein (G0S2) (13, 22).

Summary of findings

There have been several elegant studies indicating a dose-dependent inhibition of ATGL by G0S2 in HeLa cells, suggesting that in situations where lipolysis is increased through ATGL catalytic activity, it perhaps is controlled by the relative content of G0S2 decreasing to release inhibition over ATGL (13, 22). Results from Chapter 4 indicate that protein content alone does not relate to resting metabolic behavior, since there is the greatest G0S2 protein in the highly oxidative soleus muscle, which happens to correspond to greatest levels of ATGL levels. Understanding the importance of the relative ratio of G0S2-to-ATGL across these tissues demonstrated that the glycolytic white gastrocnemius did not have the greatest G0S2-to-ATGL ratio compared to the more oxidative muscles, contradictory to what would be expected if G0S2 inhibition was dictated through G0S2 protein content alone.

It was initially hypothesized that ATGL was exclusively expressed in type I fibers (8), and results from Chapter 4 indicate that ATGL is predominantly expressed in type I fibers since there was the greatest amount of ATGL in the soleus with slightly less ATGL in the mixed oxidative/glycolytic red gastrocnemius. There was however, detectable levels of ATGL in the highly glycolytic white gastrocnemius, which contains essentially no type I fibers (5). To speculate on these results, perhaps ATGL protein is exclusively expressed in oxidative fibers such that not only type I, but also type IIa fibers, accounting for minor relative protein content in the almost exclusively type IIb white gastrocnemius, which has been reported to have roughly 8% type IIa fiber composition (5).

Throughout the endurance training protocol, there was a significant decrease in body weight in the trained group when compared to the sedentary group (Chapter 5). This was consistent with the sedentary group eating more food weekly compared to the trained group. As the training progressed, body weights from both the trained and sedentary groups increased, with the sedentary groups gaining more weight, however there was no linear increase in food intake for both groups despite weekly increases in body weights. In male and female Osborne Mendel rats, there were time related increases in body weights, however only males differed in body weights due to training when compared to gender matched sedentary controls, where they had lower weights due to training, with female rats having no differences between gender matched groups (1). Interestingly, when food weight was compared, there were no differences in cumulative food intake during the twelve-hour dark cycle in either males or females regardless of training status, however during the twelve-hour light cycle, the only change that was observed was a decrease in food consumption with the endurance-trained male rats. This is suggestive of

not only a gender difference between males and females for body weights and food consumptions, also a time preference for feeding behavior, which is especially interesting since the training protocol was conducted during the start of the twelve-hour dark cycle. However, this also could be suggestive of the trained group sleeping more while the sedentary group perhaps ate. Male Sprague-Dawley rats were treadmill trained for four days a week, with the other three days sedentary, during the training period, there was a reduction in both food intake and body weight, yet during the sedentary phase, these effects were attenuated (18). In lean healthy human males, there was a short term appetite suppression following exercise which delayed the onset of feeding post workout, however there was only an observed negative energy balance observed during longer exercise bouts (9). To speculate, perhaps during the training periods, the redistribution of blood flow away from the stomach (2), to the muscle leads to a decrease in appetite.

Rodent and human training protocols cannot be directly compared since rodent training is through the use of four limbs, with human treadmill endurance training being bipedal. A strength of rodent skeletal muscle is the homogeneity of the muscle fiber types, however this is not consistent with human skeletal muscle, since human skeletal muscle is much more mixed. Following a progressive 9-week training protocol on stationary bicycles, there was an increase in vastus lateralis citrate synthase from 13.7 to 23.2 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet wt, this is similar to the changes observed in chapter 5 in the white gastrocnemius which went from 15.3 to 27.9 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet wt (6).

G0S2 as a lipolytic inhibitor

In G0S2 overexpressed HeLa cells, there was a decrease in lipolytic rates as it was observed that G0S2 localized to the lipid droplet membrane, preventing triglyceride

degradation through inhibition of ATGL. This was done perhaps through direct interaction between the patatin-like domain of ATGL with the hydrophobic region of G0S2 (22). Following knockdown of G0S2 in HeLa cells, there was an acceleration of both basal, and isoproterenol stimulated lipolysis, identifying G0S2 as a direct inhibitor on ATGL regulating the inhibition of lipolysis. In Chapter 5, it was shown that ATGL protein content increases due to endurance training. While this result alone is not novel, the discovery that there were increases across a range of metabolically diverse skeletal muscles, such that the greatest increase observed was in the white gastrocnemius, which had the least amount of ATGL protein content when compared to the soleus or the red gastrocnemius in resting sedentary muscle is novel. With CGI-58 unchanged, based on previous literature indicating that lower total G0S2 content could predict higher rates of lipolysis, it was expected that G0S2 protein content would decrease due to endurance training. However, contrary to our hypothesis, there were substantial increases in G0S2 protein content in both the oxidative soleus and red gastrocnemius with endurance training, yet there was no change in total G0S2 protein content in the soleus or red gastrocnemius following an acute lipolytic inducing contraction, suggesting that G0S2 is not a physiological dose dependent inhibitor of ATGL in skeletal muscle since there was no decrease in G0S2 protein content following a contraction protocol that significantly reduced intramuscular lipid content. In subcutaneous adipose tissue from adult males following a 72-hour fast, an increase in ATGL protein content was observed, with a decrease in G0S2 content (14). The fasting condition caused an increase in circulating free fatty acids indicating a breakdown of lipid droplets from adipose tissue, which is putatively facilitated by the decrease in G0S2 protein content. Alternatively, in the same

study, an acute bout of exercise conducted in a separate trial from the fasting condition, similar amounts of free fatty acid release was observed, with a decrease in c-peptide as well, however there were no detectable changes in ATGL or G0S2 protein content. The decrease in C-peptide following both protocols is important to note since it is postulated that G0S2 protein is modulated by circulating insulin levels (15, 22), however the decrease in insulin following an acute bout of training led to no changes in G0S2 protein content, yet following fasting there was a decrease in G0S2 protein content, suggesting alternative regulation. Although, this perhaps is tissue specific, since it has been demonstrated in skeletal muscle that before and after a hyperinsulinemic-euglycemic clamp, there was a significant increase in G0S2 mRNA expression (15), yet in 3T3-L1 mouse adipocytes stimulated with either insulin or isoproterenol, there were no acute effects altering the G0S2-to-ATGL interaction, yet following chronic exposure there was a decrease in G0S2 with an increase ATGL (22). Our results are consistent with G0S2 perhaps being a long-term regulator of ATGL, however with contradictory results as previously reported, since following training we witnessed an increase in G0S2 protein content coordinately with an increase in ATGL protein content.

The only skeletal muscle where there was no observable change in G0S2 protein content in response to endurance training was the highly glycolytic white gastrocnemius. This is interesting, because there had been a robust increase of ATGL protein content in this muscle, suggesting a tissue specific response to training.

G0S2 as a mitochondrial protein

Furthermore, there was also increased enrichment of G0S2 protein in isolated subsarcolemmal mitochondria from red gastrocnemius in response to endurance training,

although this was not altered following acute lipolytic inducing contraction. Subsarcolemmal mitochondria, compared to intramyofibrillar mitochondria, is in closer proximity to lipid droplets (17). To further complicate the matter, it has been speculated that G0S2 within the mitochondria is a positive up regulator of oxidative phosphorylation through interaction with Complex V (F_0F_1 ATPase) (10). Our data are inconsistent with this hypothesis, since it is assumed that there is greater ATP turnover during our 30-minute contraction stimulus, which did decrease intramuscular lipid content significantly, yet there was no change in acute G0S2 protein content. Separately, it is speculated that G0S2 may have an entirely separate function in the mitochondria, in that it might prevent the anti-apoptotic heterodimer of Bcl-2/Bax in turn promoting apoptosis (20). Results from both chapter 4 and 5 cannot directly comment on the function of G0S2 in the mitochondria, it is however in support that G0S2 being a mitochondrial protein. From Chapter 4, whole muscle G0S2 protein content is the greatest in the oxidative soleus and red gastrocnemius, both of which have greater mitochondrial densities compared the more glycolytic white gastrocnemius (20). Although, if this were entirely true, it would be expected that G0S2 would be greater in the red gastrocnemius compared to perhaps even the soleus, since it has the greater mitochondrial capability, as represented by citrate synthase data in both Chapter 4 and Chapter 5. Further study is required into the understanding of the function(s) of G0S2 in both whole tissue and in mitochondria.

Future studies

The results from the research in this thesis suggest that the mechanisms of regulation on ATGL are more complicated than previously suggested in cell models, at least for G0S2 inhibition on ATGL. This thesis focused entirely on adaptation responses

of ATGL, CGI-58 and G0S2 following endurance training (where it is believed that there are altered dynamics of intramuscular lipid reliance) and acute lipolytic contraction in several metabolically different skeletal muscles, as well as isolated mitochondria, without attempting to examine underlying intracellular regulation of the specific proteins.

Determining the role of G0S2 in lipolysis

Through over- and under-expressing G0S2 in HeLa cells, G0S2 has been identified as a direct 'dose-dependent' inhibitor on ATGL catalytic activity (13, 22). However, this thesis shows that G0S2 increases in oxidative tissues, which does not support this notion. Future work should explore whether this regulation remains in mammalian tissue, and if so, perhaps attempting to explore intracellular or post-translational modification on G0S2, as well as further exploring the regulation on ATGL. Recently, new phosphorylation sites have been identified on ATGL, which may alter ATGL translocation ability throughout the cell (21). This is important, since the interaction between ATGL and G0S2 may not be determined entirely through G0S2 modifications alone, perhaps regulation directly on ATGL is required to modify the ability of G0S2 to inhibit its catalytic behavior. Before a lipolytic function of G0S2 was suggested, it was identified that G0S2 has the potential to be phosphorylated by both protein kinase C and casein kinase II (16), although the physiological significance of these phosphorylation sites have yet to be ascertained. Moreover, a direct interaction through co-immunolocalization or co-immunoprecipitation can be used to determine a potential interaction between ATGL and G0S2 *in vivo.* Previously, it has been theorized that ATGL and G0S2 are bound at all times (13), which alone would suggest another level of regulation onto the inhibition of ATGL through G0S2. However, this has yet to

be determined to be a mammalian interaction, and the physiological significance has yet to be determined.

Two previous studies to date have examined G0S2 specifically within the mitochondria, and have each identified a different, almost opposing, function of G0S2. Whether G0S2 is a pro-apoptotic protein (20), or a positive regulator of oxidative phosphorylation (10), or regulators of both functions has yet to be further explored. However, understanding of G0S2 regulation might supersede this information, since specific regulation may in turn facilitate in determine specific functions of G0S2.

What may, in future be an exceptional research tool for elucidating specific protein function may be *in vivo* genome editing through a system such as CRISPR-Cas9 (23). The ability to create a tissue specific knockout animal, without the potential for lifelong endogenous adaptations may yet help indicate specific tissue function. Through skeletal muscle, or other tissue G0S2 knockout it may aid in determining loss of function protein effects.

CGI-58 function in regulating lipolysis

With CGI-58 remaining unchanged following endurance training, but both other potential regulators of lipolysis through ATGL change following training needs to be further explored. How the co-activation of ATGL occurs through interaction of CGI-58 remains relatively unexplored, however one potential hypothesis is that CGI-58 is perhaps embedded in the membrane of the lipid droplet, and facilitates ATGL exposure to its triglyceride substrate (13). CGI-58 appears to have several functions throughout the body, since mutations have been identified (up to 8) as reasons for Chanarin-Dorfman syndrome (12). Chanarin-Dorfman syndrome is characterized by an increase in

triglyceride accumulation, which is consistent with its function as a co-activator of ATGL, but patients with Chanarin-Dorfman also suffer from "ichthyosis", which is characterized as rough, scale-like skin, which to date is inconsistent with its role as a co-activator of ATGL, suggesting at least one other function of CGI-58 within the body.

Limitations

The primary limitation of this thesis is that it is underpowered, as most physiological studies are. This is important, since what we report as insignificant results, may not have a great enough power to detect any changes. Larger sample sizes could benefit future studies by being able to detect smaller changes due to any perturbation on lipolytic rates. Using information from Chapter 5, the means, standard deviations and samples size (α=0.05), we were under power, β=0.5. However, in order to reach to a desired sample size of 22 (if both α=0.05, β=0.8), would not only be financially difficult to achieve, but also ethically difficult due to the preventable use of animals.

A strength of this study was the model used, since rats are an excellent model to study variances in fiber type with having highly homogeneous muscles towards different fiber types, with also having mixed muscle. However a limitation is that information was gained only from one species, and may not translate into other mammalian physiology. As well as, males were only used for this study with many differences in metabolic rates between males and females.

Conclusion

The purpose of this thesis was to expand our understanding of ATGL, the co-activator CGI-58, and the inhibitor G0S2 protein. Since it was postulated that the

mechanism for inhibition of ATGL through G0S2 was dose dependent, we postulated that using a model of three metabolically diverse skeletal muscles, we could profile relative expression of ATGL, CGI-58 and G0S2 mRNA and protein content across these tissues based on there intramuscular reliance on fat as fuel. Logically, it was discovered that ATGL was highest in the oxidative soleus, and lowest in the glycolytic white gastrocnemius, yet paradoxically G0S2 protein content followed the same pattern of greatest expression in the oxidative soleus, with the least in the glycolytic white gastrocnemius. Examining the ratios of G0S2-to-ATGL, there was no difference between any of the muscles, suggesting more complicated intracellular mechanisms for inhibition *in vivo* of ATGL by G0S2. Following endurance training, there was an expected increase in ATGL protein content across all three skeletal muscles, with the greatest increase in the glycolytic white gastrocnemius, with an anticipated no change in CGI-58 protein content, however G0S2 protein content increased as well, interestingly however only in the oxidative soleus and red gastrocnemius, no change was observed in the white gastrocnemius, despite the most robust change in ATGL protein content. Finally, during an acute lipolytic inducing contraction, there was no change in whole muscle, or in isolated mitochondrial G0S2 protein content, further implying greater regulation of protein function since during contraction there was no decrease in G0S2 protein which was hypothesized to facilitate lipolysis through the relief of inhibition of ATGL.

Summary

The regulation of ATGL via co-activation and inhibition by CGI-58 and G0S2, respectively, is still not relatively well understood. Currently, it does appear that CGI-58-to-ATGL interaction is crucial for the up regulation of ATGL, and that it appears that

CGI-58 is saturated in skeletal muscle since no change occurs following endurance training, however this may well be explained in the future by further understanding the nature of the interaction between ATGL and CGI-58. The inhibition of ATGL through G0S2 has yet to be observed in physiological tissues, however several interesting cell models have indicated G0S2 as a dose dependent inhibitor of ATGL through increases of G0S2 decrease ATGL activity. However, our results do not agree with this hypothesis, since not only were there greater proportionate amounts of G0S2 to ATGL in the more oxidative skeletal muscles, but there was also an increase in G0S2 protein content in these oxidative tissues due to training. Although unknown, perhaps the increase in G0S2 protein content due to endurance training in skeletal muscle is perhaps a tissue specific mechanism for increasing control of lipolytic rates. The results from these studies have begun a foundation of examining this triad of proteins in physiological systems to better understand the regulation of lipolysis, and perhaps elucidating the true function of the G0S2 protein not only in whole tissue, but mitochondrial specific functions as well.

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